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Cover illustration taken from a scanned microarray image.
Adapted by Fuensanta Candela & Paul Brady.

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Prenatal Chromosomal Microarray Analysis and Identification of Genetic Variants in Congenital Diaphragmatic Hernia (CDH)

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List of Abbreviations

AF	Amniotic Fluid
AMA	Advanced Maternal Age
AOH	Absence of Heterozygosity
Array CGH	Array Comparative Genomic Hybridisation
ASD	Autistic Spectrum Disorder
ASD	Atrial Septal Defect
BAF	B-Allele Frequency
CDH	Congenital Diaphragmatic Hernia
CGH	Comparative Genomic Hybridisation
CNV	Copy Number Variation
CVMs	Cardiovascular Malformations
CVS	Chorionic Villus Sample
DD	Developmental Delay
DGV	Database of Genomic Variants
DLR	Derivative Log Ratio
DMR	Differentially Methylated Region
DNA	Deoxyribonucleic Acid
ECM	ExtraCellular Matrix
ESP	Exome Sequencing Project
FBMs	Foetal Breathing Movements
FETO	Fetoscopic Endoluminal Tracheal Occlusion
FDR	False Discovery Rate
FISH	Fluorescent *in situ* Hybridisation
GPI	Glycosylphosphatidylinositol
GWAS	Genome-Wide Association Study
HPO	Human Phenotype Ontology
IBD	Identical by Descent
ID	Intellectual Disability
Indel	Insertion / Deletion
iPOP	integrated Personal Omics Profile
Kb	Kilobases
LDDB	London Dysmorphology Database
LHR	Lung to Head Ratio
LOH	Loss of Heterozygosity
Mb	Mega-bases
MCA	Multiple Congenital Anomalies
MCC	Maternal Cell Contamination
MDR	Minimal Deleted/Duplicated Region
MeDIP	Methylated DNA Immuno-precipitation
MLPA	Multiplex Ligation-dependent Probe Amplification
mRNA	messenger RNA
MSCs	Mesenchymal Stem Cells
NIPD	Non-invasive Prenatal Diagnosis
NIPT	Non-invasive Prenatal Testing
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
PPF	Pleuroperitoneal Fold
qf-PCR	Quantitative-fluorescent PCR
qPCR	Quantitative PCR
RA	Retinoic Acid
RNA	Ribonucleic Acid
RPKM	reads per kilobase per million reads
SD	Standard Deviation

SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SRO	Smallest Region of Overlap
STR	Short Tandem Repeat
TAPVR	Total Anomalous Pulmonary Venous Return
TL	Tracheal Ligation
TO	Tracheal Occlusion
UPD	Uniparental Disomy
US	Ultrasound
VAD	Vitamin A Deficient
VOUS	Variant of Uncertain Significance
VSD	Ventricular Septal Defect
WGA	Whole Genome Amplification

Chapter 1

Introduction

Chapter 1. Introduction

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Vermeesch, J., Brady, P.D., Sanlaville, D., Kok, K., Hastings, R. (2012). Genome-wide arrays: Quality criteria and platforms to be used in routine diagnostics. Human Mutation, 33(6), 906-915.

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Brady, P.D., Devriendt, K., Deprest, J., Vermeesch, J. (2012). Array-based approaches in prenatal diagnosis. In: Feuk L. (Eds.), Genomic Structural Variants: Methods and Protocols. Series: Methods in Molecular Biology vol. 838, Chapt. 7. Springer Science, 151-171.

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1.1 Prenatal Genetic Diagnosis

1.1.1 Historical Introduction

Karyotyping (from the Greek *karyon*, meaning kernel, seed or nucleus), describes the visual assessment of chromosome number and appearance in the cells under a light microscope. In 1959, Lejeune *et al* correctly identified that an additional copy of chromosome 21, trisomy 21, was the cause of Downs syndrome in humans (Lejeune et al., 1959). In 1966, Steel and Breg demonstrated that the chromosomal constitution of the foetus could be determined from the analysis of cultured cells obtained from amniotic fluid sampling (Steel and Breg, 1966). The following year, Jacobsen and Barter reported the first prenatal diagnosis of a chromosomal abnormality (Jacobson and Barter, 1967). Following the continued realisation that chromosome abnormalities are a major cause of congenital anomalies and developmental disorders, karyotyping became the gold standard for the genome-wide detection of genomic imbalances in prenatal diagnosis. Though karyotyping allows for the genome-wide detection of large chromosomal abnormalities and translocations, it has a number of inherent limitations; (i) the culturing of cells requires a minimum of 8-10 days, and can lead to a selective advantage of specific cell lines; (ii) the resolution is limited to 5-10Mb, at very best; and (iii) the visual screening for numerical or structural chromosome anomalies requires skilled analysts and is time consuming.

In the presence of specific ultrasound findings, a single gene (monogenic) disorder may be suspected as a cause. This directs the appropriate genetic testing to analysis of a specific gene which may be undertaken by PCR based methods or conventional Sanger sequencing. Alternatively, a prior family history may lead to predictive testing of the foetus for the respective mutation(s). One of the most frequent PCR based tests applied in the prenatal setting is for identification of commonly observed mutations in the CFTR gene which causes the autosomal recessive disorder cystic fibrosis. The finding of an echogenic bowel on prenatal ultrasound suggests an intestinal obstruction caused by abnormal meconium. Among fetuses who have hyperechogenic bowel on prenatal ultrasound, about 1 in 10 will have CF. One in 25 people of European descent are carriers for a mutation in the CFTR gene, with the ΔF508 allele being the most frequently observed mutation.

1.1.2 The Emergence of Molecular Cytogenetics

As genetic techniques have advanced there has been a natural progression in applying DNA-based molecular genetics techniques to conventional cytogenetic investigations leading to the emergence of ‘molecular cytogenetics’, bringing the two disciplines closer than ever. Several novel molecular biology techniques have been applied to rapid aneuploidy detection, notably, quantitative-

fluorescent PCR (qf-PCR) (Adinolfi et al., 1997; Mann et al., 2001) and multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002; Schouten and Galjaard, 2008) which are able to provide a result in 1-2 days. These techniques apply DNA-based methods to determine genomic copy number changes, but have the same locus-specific disadvantages as for FISH (Shaffer and Bui, 2007; Wang and Cui, 2010).

1.1.2.1. FISH

Fluorescent *in situ* hybridisation (FISH) analysis utilises fluorescently labelled DNA probes complementary to the locus of interest which are hybridised to the respective chromosomal location and visualised with fluorescent microscopy. In the normal diploid situation, 2 fluorescent signals are observed per cell examined whereas deletions show only a single signal and duplications three signals. The vast majority of prenatal genetic diagnosis is aimed at the detection of a limited number of aneuploidies due mainly to the increased risk associated with advanced maternal age. FISH probes targeted to loci on chromosomes 21, 18, 13, X & Y became the method of choice due to the ability to provide a rapid result in only 2-3 days for the common trisomies and sex chromosome aneuploidies (termed rapid aneuploidy detection) (Philip et al., 1994; Bryndorf et al., 1996; Bryndorf et al., 2000; Tepperberg et al., 2001). Furthermore, specific ultrasound anomalies, or prior family history, may direct targeted analysis to a specific genomic locus. One of the most common targeted FISH tests requested in the prenatal setting is for the detection of 22q11 deletion (DiGeorge / velocardiofacial syndrome) in the presence of congenital heart defects and /or cleft palate.

1.1.2.1. qf-PCR

qf-PCR utilises multiplexing of polymorphic STR markers located on chromosomes of interest to determine aneuploidy status. Fluorescently labeled primers are used for PCR amplification of individual STR markers which are then characterised using fragment analysis by capillary electrophoresis allowing for the detection of normal or aneuploidy status. Informative alleles will show an allelic ratio of 1:1, whereas trisomic samples will show the presence of an additional allele as three peaks in a 1:1:1 ratio or as two peaks in a 2:1 or 1:2 ratio. Multiplexing of STR markers can thus provide a single tube assay for chromosomes 13, 18, 21, X & Y.

1.1.2.2 MLPA

MLPA utilises two separate oligonucleotides, which hybridise to immediately adjacent target genomic sequences. If both oligonucleotides hybridise to their adjacent targets they are ligated and subsequently exponentially amplified during the PCR reaction. The numbers of probe ligation products are thus a measure of the number of target sequences in the sample investigated allowing for detection of duplications or deletions of the respective locus. Similar to qf-PCR capillary electrophoresis is used to separate and quantify the differently sized amplification products in a single run.

1.1.3 Chromosomal Microarrays

Chromosomal microarrays allow for 'molecular karyotyping' (Vermeesch et al., 2005), and overcome the resolution, locus-specific, and time limitations of the aforementioned techniques. Chromosomal microarrays provide a genome-wide screen for gains and losses of genomic regions at a far superior resolution to karyotyping; the resolution is only dependent upon the number and size of the targets on the array. Current diagnostic platforms typically achieve resolutions of several kilobases to several hundreds of kilobases. Chromosomal microarrays have revolutionised genetic diagnosis over recent years and have led to the discovery of a number of new recurrent microdeletion and microduplication syndromes (Sharp et al., 2006; Mefford et al., 2007; Shaffer et al., 2007; Mefford et al., 2008; Rudd et al., 2009; Wat et al., 2010; Dittwald et al., 2013). Aside from those recurrent microdeletion / microduplication syndromes, novel copy number variations (CNVs) are also identified. The 'genotype first' approach (Mefford, 2009) allows to define a 'minimal deleted/duplicated region' (MDR) or 'smallest region of overlap' (SRO) by comparing overlapping CNVs from multiple patients with similar phenotypic features. Examples include the SRO at 15q26 and 1q41-42, both of which are associated with multiple congenital anomalies (MCA) including congenital diaphragmatic hernia (CDH) in some affected individuals (Slavotinek et al., 2006; Klaassens et al., 2005; Rosenfeld et al., 2011; Kantarci et al., 2010; Shaffer et al., 2007).

There are two types of array technologies available; array CGH (comparative genomic hybridisation); and SNP (single nucleotide polymorphism) arrays.

1.1.3.1 Array CGH

For array CGH, patient DNA and reference DNA are differentially labelled with fluorescent dyes and co-hybridised to complementary oligonucleotide targets, with known genomic coordinates, which are printed, i.e. an array, on a solid substrate, typically a glass slide. The fluorescent signal intensity

ratio of patient DNA to reference DNA from individual targets is measured using a laser scanner, and the results can be plotted and visualised by the respective consecutive genomic locations of the target oligonucleotides, allowing gains or losses of chromosomal material in comparison to a normal reference to be determined. The schematic shown in Figure 1.1.1 provides an overview of the workflow, and Figure 1.1.2 shows an image of a genomic array result in which both deleted and duplicated regions are visualised.

1.1.3.2 SNP Arrays

SNP arrays were originally designed to detect common (>1% in the population) single nucleotide polymorphisms (SNPs) and were mainly used in genotyping individuals for studies aimed at genome-wide association studies (GWAS) of many common multifactorial diseases (Klein et al., 2005; Wellcome Trust Case Control Consortium, 2007; Sladek et al., 2007). In addition to SNP typing, these platforms can also be used to perform copy number analysis by comparing the patient target signal intensities to those of a reference dataset, as opposed to the comparative dual hybridisation used in array CGH. Gains & losses of genomic regions can therefore be detected as is the case for CGH arrays. SNP array manufacturers have added additional oligonucleotide targets not aimed at detecting SNPs, but to provide an even coverage of the genome for improved CNV detection. SNP identification is performed by calculation of the B-allele frequency (BAF), a measurement of the presence of allele 'B' and allele 'A'. Homozygous sites are called as AA (BAF=0) or BB (BAF=1), whilst heterozygous sites are called as 'AB' (BAF=0.5). The B-allele frequency (BAF) of the respective SNP targets also allows SNP arrays to detect copy neutral LOH (or absence of heterozygosity, AOH), uniparental disomy (UPD), and regions identical by descent (IBD). However, whilst SNP arrays will detect uniparental isodisomy, parental samples are required for the detection of uniparental heterodisomy. Figure 1.1.3 provides a representation of how different types of copy number and SNP data from genomic arrays can be plotted and visualised.

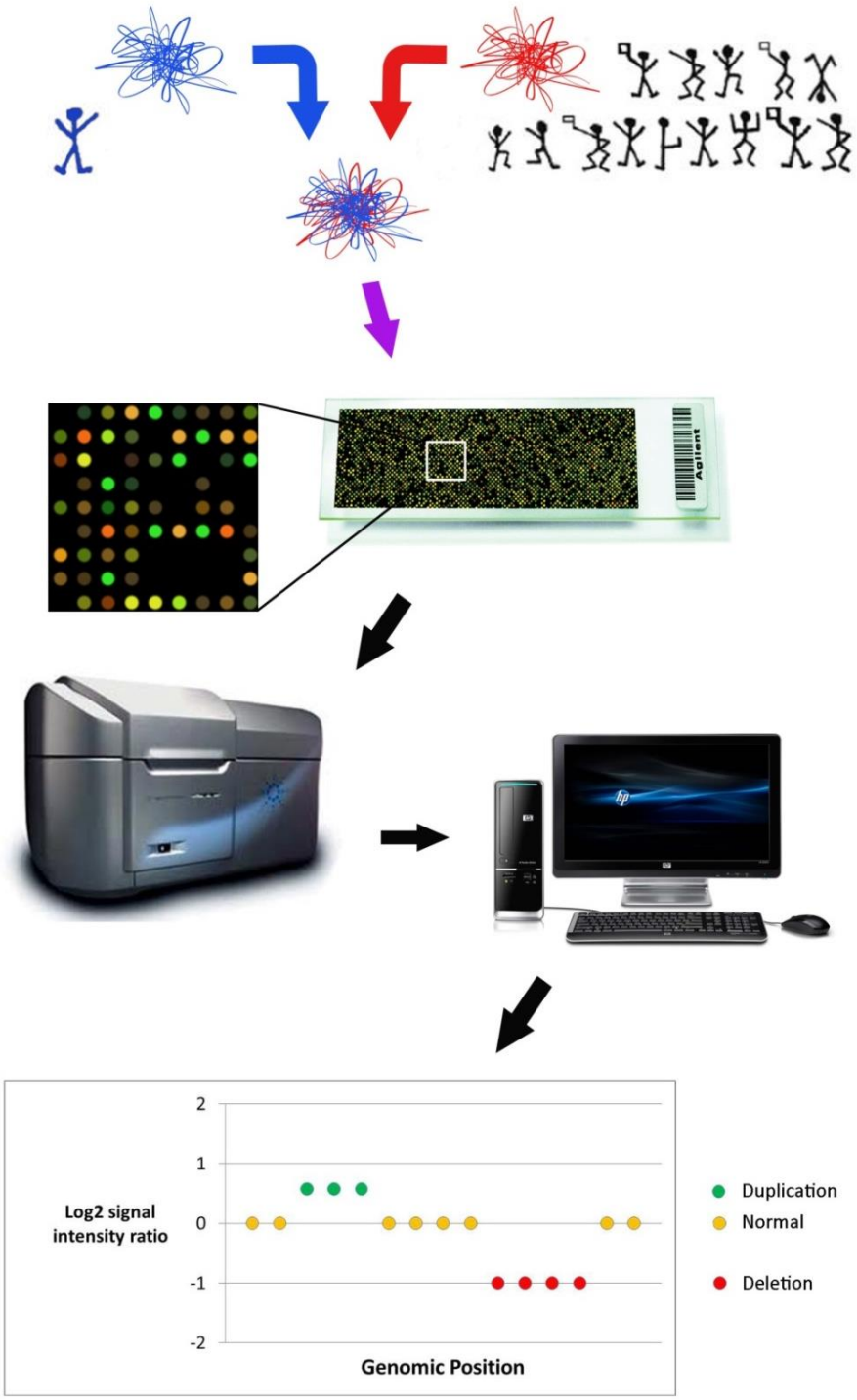

Figure 1.1.1. An overview of the array workflow is presented. Patient DNA (blue, top left) and Reference DNA (red, top right) are fluorescently labelled with Cy5 and Cy3 dyes, respectively. Equal quantities of DNA are mixed and co-hybridised to complementary oligonucleotide probes printed onto a microarray. After washing unbound DNA from the array, a laser scanner is used to generate a high resolution TIFF image with the fluorescent signal intensities. This data is further processed and plotted by the genomic position of the oligonucleotide probe on the x-axis and by the patient:reference normalised log2 signal intensity ratio on the y-axis.

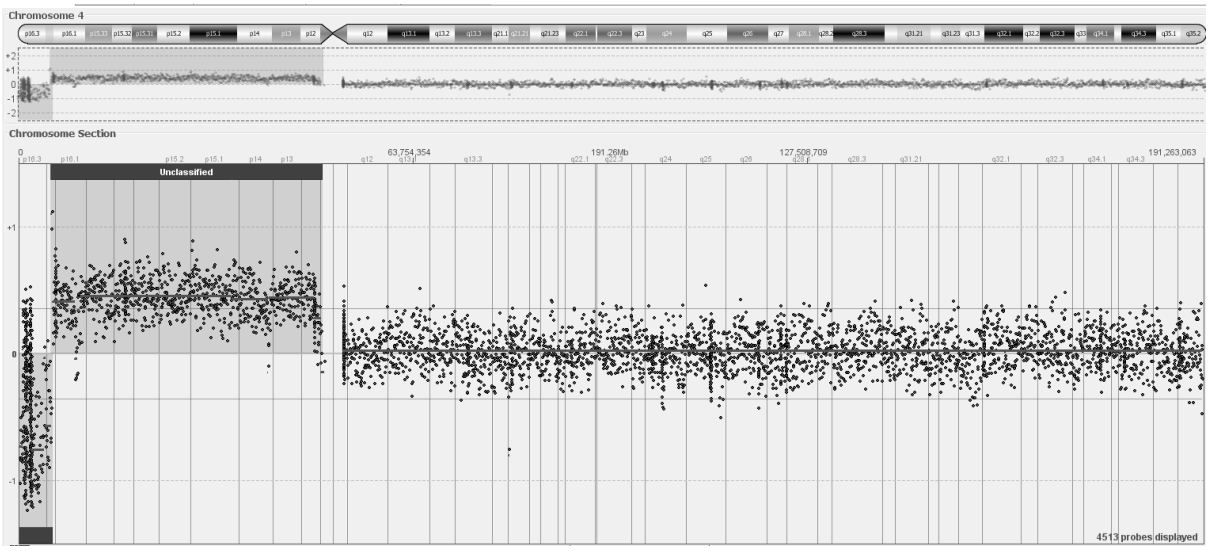

Figure 1.1.2. Displays an image from a genomic array used for array CGH analysis of a prenatal sample. Chromosome 4 is shown, where individual targets are plotted by genomic position on the x-axis and by normalised Log_2 signal intensity ratio on the y-axis. Deletion of 4p16.3 (with values approaching -1) as well as duplication of the remaining 4p arm (with values approaching 0.58) can be seen highlighted as aberrant regions by the analysis software, as a result of an inverted duplication deletion of chromosome arm 4p. The 4q chromosome arm displays normal copy number, with Log_2 ratios close to zero.

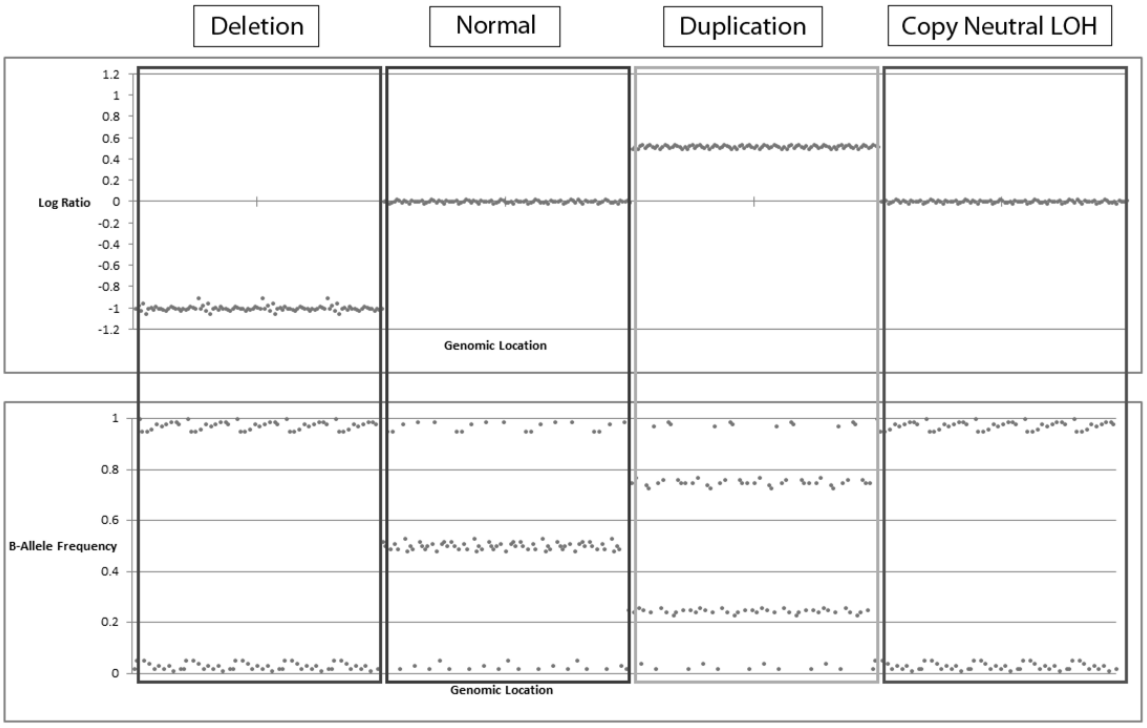

Figure 1.1.3. A representation of the types of data plots from genomic arrays.
Top graph displays a typical plot of array CGH data, where individual targets are plotted by genomic position on the x-axis, and by the normalised Log R signal intensity ratios on the y-axis. Bottom graph displays a typical plot of the B-allele frequency (BAF) from SNP arrays experiment, with individual SNP targets plotted by

genomic position on the x-axis also, and with the B-allele frequency plotted on the y-axis. The plots display the expected values for both copy number and SNP targets for genomic regions which show (from left to right) deletion, normal copy number, duplication, or copy neutral LOH (e.g. UPD). For the analysis of copy number, the expected normalised Log R signal intensity ratio values are -1, zero, and +0.56 for deletion, normal, and duplication, respectively. For SNP analysis, deletions display only homozygous regions (AA or BB), normal regions display both homozygous and heterozygous sites (AA, AB, BB), and duplications display skewed BAFs indicating the presence of an additional allele (AAB and ABB). Also shown is an example of copy neutral LOH, apparently normal when only performing copy number analysis, but displaying LOH for the same region indicating UPD.

1.1.4 Application of Chromosomal Microarrays to Genetic Diagnosis

Chromosomal microarrays were shown in a number of studies to increase the diagnostic yield to as much as 15-20% for individuals with multiple congenital anomalies (MCA), and unexplained developmental delay (DD) / intellectual disability (ID), and autistic spectrum disorders (ASD) (Menten et al., 2006; Stankiewicz and Beaudet, 2007; Sagoo et al., 2009), as opposed to ~3-5% by conventional karyotyping (excluding trisomy 21, and other recognisable chromosomal syndromes). Many recurrent microdeletion / microduplication syndromes, previously unidentifiable by karyotype, were discovered by the use of chromosomal microarrays (Sharp et al., 2006; Mefford et al., 2007; Shaffer et al., 2007; Mefford et al., 2008; Rudd et al., 2009; Wat et al., 2010; Dittwald et al., 2013). These findings along with increased use in diagnostic laboratories led to the international consensus statement from the scientific and medical community that chromosomal microarray analysis be offered diagnostically as the first-tier test for this cohort of patients (Miller et al., 2010), and the formation of the ISCA consortium (The International Standards For Cytogenomic Arrays Consortium; https://www.iscaconsortium.org/).

With the introduction of chromosomal microarrays into routine postnatal diagnostic use, prenatal diagnosis was left lagging behind somewhat. Clearly, genome-wide screening for submicroscopic CNVs by chromosomal microarray analysis is an attractive alternative in the prenatal diagnostic setting also, providing; (i) detection of all the known recurrent microdeletion / microduplication syndromes (at a resolution of ~400kb), (ii) a more precise delineation of rearrangement breakpoints and thus gene content, and (iii) with no need for cell culture, a faster report time (3-5 days is achievable). To this end, several early studies demonstrated the use of different types of genomic arrays for prenatal diagnosis with a variety of sample types. In an early proof of principle experiment, Rickman *et al* (Rickman et al., 2006) demonstrated the feasibility of performing array-based comparative genomic hybridisation (array CGH) for prenatal diagnosis on DNA extracted from uncultured amniotic fluid (AF) cells. Cell-free fetal DNA in AF supernatant has also been shown to be suitable for performing array CGH (Larrabee et al., 2004; Miura et al., 2006; Lapaire et al., 2007). In

addition, whole genome amplification (WGA) has been applied to small quantities of DNA to providing results from limited amounts of starting material (Bi et al., 2008).

1.1.5 Detection of mosaicism

Mosaicism can be detected with the use of genomic arrays (Scott et al., 2010; Hoang et al., 2011; Valli et al., 2011) and does not represent an inherent limitation of genomic arrays. However, the level at which mosaicism can be detected is dependent on various quality parameters, mainly the standard deviation (SD) or derivative Log ratio (DLR) spread and the dynamic range, as opposed to the absolute number of cells counted with karyotyping or FISH. Maternal cell contamination (MCC) can be a confounding factor in prenatal diagnosis, and will reduce the dynamic range of true imbalances (which will appear to be in mosaic form).

1.1.6 Limitations of Microarrays

Inherent to the technique, balanced chromosomal rearrangements (balanced translocations and inversions) are not identified by genomic arrays. When a balanced rearrangement is detected prenatally on karyotype, the parents are usually tested and if the same rearrangement is present in the 'normal' parent then the translocation is typically considered likely benign. However, if the rearrangement is *de novo*, genetic counselling is more challenging, and the empiric risk for developmental defects has been estimated to be 6% (Warburton, 1991). The study of Giardino *et al* (Giardino et al., 2009) on rates of *de novo* balanced chromosome rearrangements in prenatal diagnosis reports frequencies of 0.09%, 0.08%, and 0.05% for amniotic fluid (AF), chorionic villus sample (CVS) and foetal blood samples, respectively.

Interestingly, array CGH analysis of patients with developmental anomalies and apparently balanced translocations has revealed causal submicroscopic gains and losses which disrupt genes at the translocation sites, or affecting a chromosome not involved in the translocation (De Gregori et al., 2007; Baptista et al., 2008; Schluth-Bolard et al., 2009). The study of De Gregori et al (De Gregori et al., 2007), found that ~40% of patients with a 'chromosomal phenotype' and an apparently balanced translocation were in fact unbalanced, and a similar study by Schluth-Bolard et al (Schluth-Bolard et al., 2009), confirms the figure of ~40% of apparently balanced chromosome rearrangements being in fact unbalanced. In light of these findings, genomic arrays can provide important information in cases of apparently balanced translocations. However, karyotyping and FISH remain essential tools for the identification of truly balanced rearrangements as well as for visualization of chromosomal structure and location at the microscopic level.

In addition to truly balanced rearrangements, triploidies (69,XXX and 69,XXY) and tetraploidies are not detectable by array CGH. However, the use of DNA from a patient with Klinefelter syndrome (47,XXY) will result in aberrant X and Y chromosome intensity ratios, enabling the detection of XXX triploidies and tetraploidy (Ballif et al., 2006). An exception to this is the use of SNP arrays which can allow for the detection of triploidy (Tyreman et al., 2009).

1.1.7 Interpretation of CNVs in the prenatal setting:

The introduction into routine prenatal diagnosis has remained slow since genomic arrays have the potential to reveal 'incidental' findings, including; (i) pathogenic CNVs exhibiting variability in phenotypic expression or disease penetrance, and for which the future phenotype cannot therefore be predicted, (ii) CNVs which are unrelated to the principal referral reason but which may have clinical consequences for the future child or the parents and additional members, and (iii) CNVs for which the clinical significance is currently not known – becoming more commonly known as Variants of Uncertain Significance (VOUS). This naturally creates challenges for genetic counselling, particularly in the prenatal setting (Beaudet, 2010; Dondorp et al., 2012; McGillivray et al., 2012; Wapner et al., 2012a). More specifically, it is the detection of VOUS in the prenatal setting that has been one of the major criticisms (Kohane et al., 2006; Shuster, 2007). Early studies have estimated an increase in diagnostic yield above karyotyping alone of over 5%, with the incidence of VOUS in the region of 1-2% (Coppinger et al., 2009; Hillman et al., 2011; Shaffer et al., 2008; Strassberg et al., 2011; Van den Veyver et al., 2009; Hillman et al., 2013; Wapner et al., 2012b).

1.2 Congenital Diaphragmatic Hernia

1.2.1 The Diaphragm

The diaphragm is a 'parachute' shaped structure which has several roles; (i) it forms a physical barrier separating the thoracic cavity from the abdominal cavity; (ii) it assists with respiration by contraction of the muscular portion thus enlarging the thoracic cavity, reducing intra-thoracic pressure and drawing air into the lungs. Upon relaxation, air is exhaled by elastic recoil of the lung in conjunction with the abdominal muscles; and (iii) it assists several non-respiratory functions, such as expelling vomit, faeces, and urine from the body by increasing intra-abdominal pressure, and preventing acid reflux by exerting pressure on the esophagus as it passes through the esophageal hiatus. The mature diaphragm is show in Figure 1.2.1, from which it can be seen that a muscular rim surrounds a central area of connective tissue, with three necessary passages for the oesophagus, aorta, and inferior vena cava. The foramen of Bochdalek and foramen of Morgagni are labelled, and the location of the common posterlateral Bochdalek diaphragmatic hernia is highlighted.

The development of the normal diaphragm is still poorly understood. Perry et al have recently reviewed possible hypotheses on the evolutionary origins of the mammalian diaphragm (Perry et al., 2010). An early 'muscular hypothesis proposes that the diaphragm is primarily muscular in origin, originating by the lung invading the space between the rib cage and existing body wall musculature. The central tendon, lacking musculature, is caused by muscle atrophy. However, increasing evidence from a variety of sources suggests that the historical view of diaphragm development, mainly based upon histological sections, is incorrect. It has become apparent that the opposite is true; the diaphragm originates from a membranous structure, which becomes secondarily muscularised through development. Clugston et al investigated the embryogenesis of the pleuroperitoneal folds (PPFs) in the rodent model of CDH and in archived human embryo sections providing support for a defect in the developing PPFs as the underlying cause of the subsequent diaphragm defect later in development (Clugston et al., 2009). Interestingly, the authors identify that PPF development occurs at around 4-6 weeks gestation in humans, and thus the potential origin of CDH is earlier in gestation than previously thought. Recently, Mayer et al performed scanning electron microscopy of the developing diaphragm in a normal rodent and nitrofen rodent model of CDH adding further support to the view that the normal diaphragm mainly develops from the posthepatic mesenchymal plate or pleuroperitoneal folds (PPFs), and abnormal development of these primordial tissues results in a diaphragmatic defect (Mayer et al., 2011).

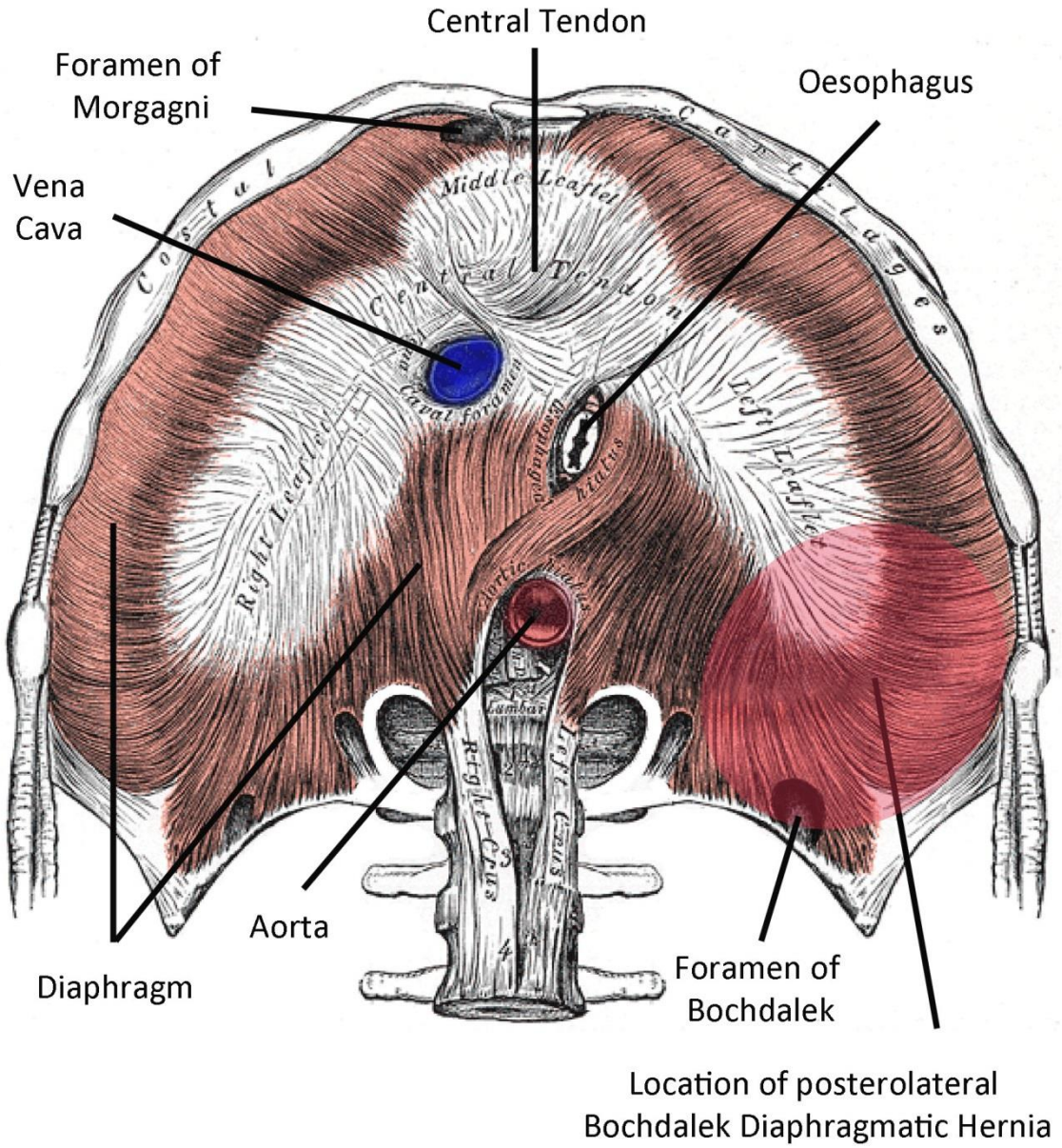

Figure 1.2.1. The Diaphragm viewed from below. Adapted from Grays Anatomy Plate 391 (sourced from; http://en.wikipedia.org/wiki/File:Gray391.png under the creative commons licence).

1.2.2 The Diaphragm Abnormality; CDH

Congenital Diaphragmatic hernia (CDH) is a defect in the development of the diaphragm leading to a 'hole' through which the abdominal contents are able to herniate, resulting in pulmonary hypoplasia due to lack of available space for the lungs to properly develop and grow, Figure 1.2.2. It is the variable degrees of pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality and most of the morbidity observed for this severe birth defect. In humans the incidence of CDH varies from 1.7 to 5.7 per 10,000 live-born infants depending on the study

population (Kotecha et al., 2011; Torfs et al., 1992; Skari et al., 2000). CDH can be anatomically divided into three main subtypes; a posterolateral 'Bochdalek' hernia representing around 70% of cases, an anterior 'Morgagni' type, accounting for around 27% of cases, and a central septum transversum hernia which totals around 3% of cases. The vast majority of hernias occur on the left side (85%), whilst the remainder are right sided (13%) or bilateral (2%) (Torfs et al., 1992; van Loenhout et al., 2009; Pober, 2007). CDH occurs as an isolated defect in around 50% of cases, or as non-isolated CDH for the remainder in which additional congenital malformations are present (Stoll et al., 2008). Non-isolated CDH is associated with abnormalities in a number of other systems including; cardiovascular system (27.5%), urogenital system (17.7%), musculoskeletal system (15.7%), and central nervous system (9.8%) (Stoll et al., 2008).

1.2.3. Current Prenatal Therapy by Fetoscopic Endoluminal Tracheal Occlusion (FETO)

Since the lung growth and function are the major factors responsible for the neonatal mortality and much of the morbidity observed in CDH, methods to correct, or at least minimise, these 'secondary' but potentially life-threatening effects in utero have been developed. The Fetal Medicine Unit of UZ Leuven (led by Professor Deprest) in collaboration with teams in London (Professor Nicolaides) and Barcelona (Professor Gratacos) have pioneered the use of FETO to accelerate lung growth and function in the moderate-severe group of CDH foetuses (measured from the lung to head ratio, LHR) (Jani et al., 2009; Deprest et al., 2011). This technique involves the insertion of a balloon into the trachea of the foetus as show in Figure 1.2.3. The foetal lungs produce fluid which accumulates in the airways, because the vocal cords at the top of the windpipe prevent it from flowing out. This fluid accumulation increases the pressure inside the lungs and stretches the airways. Foetal breathing movements (FBMs) are an essential aspect of lung development and maturation in readiness for birth and respiration, in which lung fluid escapes because of the higher pressure within the lungs. This cyclical pressure change in the airways provides the mechanotransduction signals to the lungs for necessary growth and differentiation in preparation for birth.

It is important to understand the role genetics has to play in cases that are today considered as 'isolated' CDH. Since these patients, when they also have a poor prognosis, are the focus of foetal therapy, it is this cohort of isolated CDH patients who are likely to benefit most from future therapeutic targets. Improving our understanding of the pathogenesis of CDH will reveal novel targets and pathways for future foetal therapies.

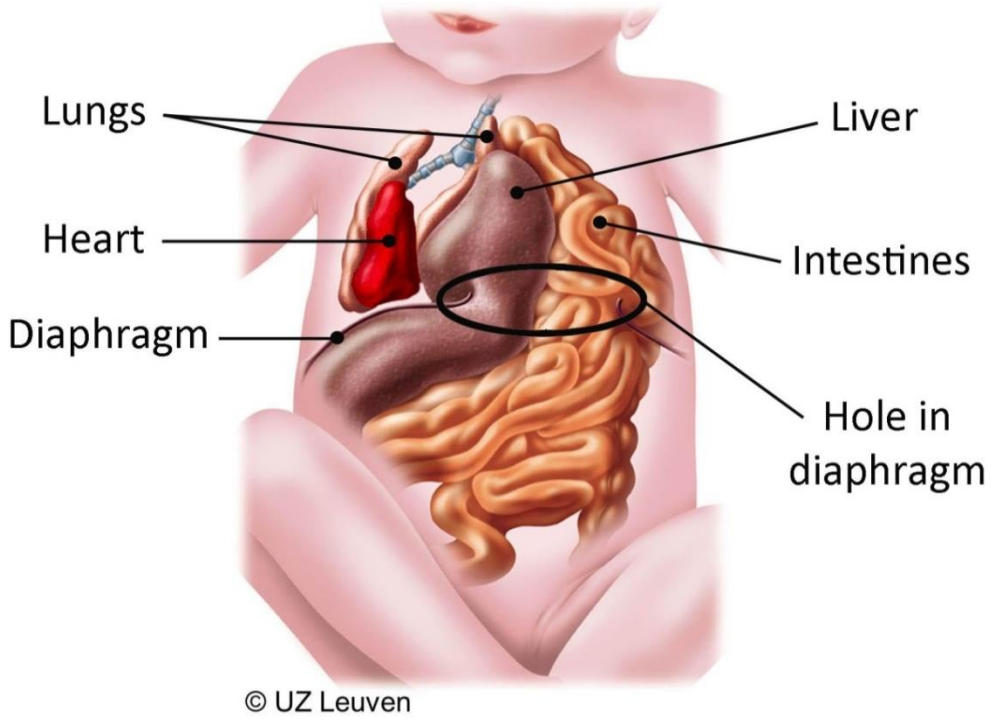

Figure 1.2.2. Congenital diaphragmatic hernia.
The illustration shows a left-sided CDH in a newborn. The hole on the left-side of the diaphragm allows the abdominal organs such as the intestines and liver to herniate and grow into the chest cavity, reducing the available space for the lungs and heart which are displaced, with the ipsilateral lung most severely affected.

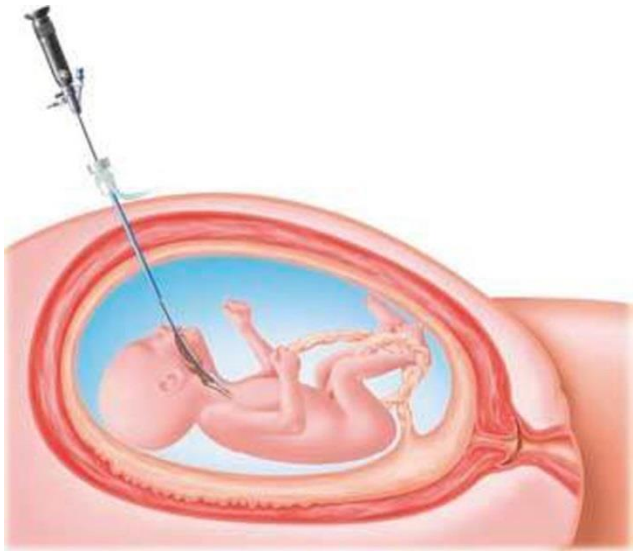

Figure 1.2.3. Illustrates the FETO procedure (adapted from www.totaltrial.com, ©UZ Leuven).
A 5mm incision is made in the maternal skin and a 3.3mm sheath is entered into the uterus under ultrasound guidance. Through this sheath a fetoscope is inserted in the uterus allowing the surgeon to visualise the foetus inside the uterus on a video screen. The foetal mouth is identified initially, and at the tracheal bifurcation into two branches for the two lungs, a small balloon is inserted. The balloon is inflated and detatched to leave in place, the surgical instruments withdrawn, and a single stitch is used to seal the entry hole. After a short hospital stay of 1 or 2 days, the mother is allowed to leave the hospital.

1.2.4 Genetics of CDH

There are more than 70 syndromes in which diaphragmatic hernias have been observed (Slavotinek, 2007). However, whether CDH has a genetic cause for all of these disorders or is merely an incidental occurrence with unique genetic aetiology in addition to the other observed malformations is uncertain. For a number of these disorders a single gene has been identified. These include genes for transcription factors, molecules involved in cell migration, and extracellular matrix components (Bielinska et al., 2007). The elucidation of the genetic mechanisms by which these genes exert their phenotypic effects may shed light on the cause of isolated diaphragmatic defects and the specific role that these genes play in diaphragm development.

Non-isolated CDH may occur as part of a recognised genetic syndrome for which a single causal gene may be identified, for example; STRA6 in Matthew-Wood syndrome (Golzio et al., 2007; Pasutto et al., 2007; Chassaing et al., 2009; Segel et al., 2009), WT1 in Denys-Drash syndrome (Devriendt et al., 1995; Antonius et al., 2008), LRP2 in Donnai-Barrow syndrome (Kantarci et al., 2007; Kantarci et al., 2008), or EFNB1 in craniofrontonasal syndrome (Wieland et al., 2004; Vasudevan et al., 2006). Alternatively, non-isolated CDH may be associated with rearrangements in specific genetic loci, including 8p23.1 (Faivre et al., 1998; Wat et al., 2009), and 15q26 (Klaassens et al., 2005; Slavotinek et al., 2006; Klaassens et al., 2007), or with a clinically recognised syndrome of currently unknown genetic cause such as Fryns syndrome (Fryns et al., 1979; Fryns et al., 1989; Neville et al., 2002; Slavotinek, 2004). While much information has been gained from the study of syndromic CDH, the exact genetic causes of isolated CDH remain largely unknown. The use of techniques such as chromosomal microarrays to search for submicroscopic CNVs in isolated CDH patients may help to unravel the contribution of genetic factors in cases of isolated CDH.

1.2.5 Animal Models

A wealth of information has been obtained from teratogen- or surgically-induced CDH animal models, as well as from genetic and transgenic models of CDH. Much research efforts, particularly those using surgically induced CDH models, have focussed on the pathogenesis of the altered pulmonary airway and vascular development. This is understandable since it is the lung hypoplasia and pulmonary hypertension which are the factors responsible for the immediate mortality and contribute largely to the long term morbidity often seen in CDH patients. Aside from the disadvantages of using teratogen-induced models to study the genetics of CDH, the nitrofen-induced CDH model has provided a better understanding of normal and abnormal diaphragm development at the anatomical level. Furthermore, the nitrofen model has also highlighted differences in gene

expression in the diaphragm and lungs, as well as providing further evidence of the retinoic acid pathway as having a causative role in the pathogenesis of CDH. The mechanism of action of nitrofen which was for long uncertain has now been demonstrated to occur by inhibition of the rate-limiting enzymes involved in retinoic acid synthesis, most likely by suppression of RALDH2 activity and thus retinoid signalling (Mey et al., 2003; Noble et al., 2007; Clugston et al., 2010).

Among the most interesting research developments using teratogen-induced animal models is the recent investigation of abnormal retinoid signalling from Clugston *et al* (Clugston et al., 2010). In this study, a number of teratogens were used to induce CDH in RARE-lacZ transgenic mice providing further evidence that disruption of retinoid signalling causes CDH, and is not specific to the nitrofen model alone. In common with other studies, dietary supplementation of retinoic acid (RA) was shown to reduce the incidence of diaphragm defects from 58.7% to 1.3% supporting the concept of possible therapeutic intervention through maintaining normal retinoid levels, and thus retinoid dependent signalling, during development. Expression analysis in the pleuroperitoneal folds (PPFs) showed that proteins involved in the retinoid signalling pathway are expressed within this structure which will be discussed later. Of particular interest, this study generated a novel CDH model using the pan-RAR antagonist BMS493 to pharmacologically block retinoic acid receptor (RAR) signalling. This model produced a Bochdalek CDH phenotype, which is the most common type observed in humans. Furthermore, the timing of teratogen exposure correlated with the side of the diaphragm defect, i.e. early administration (E8-E9) generated predominantly left-sided CDH, whilst late administration (E11-E13) generated predominantly right-sided CDH, and exposure on E10 produced left and right-sided CDH as well as bilateral CDH. These results are suggestive of different critical time periods in development of the left or right side of the diaphragm.

Historical evidence from vitamin A deficient (VAD) models have long implicated the retinoic acid pathway as having an important role in diaphragm development and therefore being a potential target for foetal therapy. More recently, genetic models have demonstrated the phenotypic effects of faults in specific genes, and further research in these models and the development of novel genetic models has the potential to pinpoint the roles that individual candidate genes play in diaphragm and lung development. Table 1 summarises the genetic mouse models in which diaphragm defects have been observed.

ID	Gene Symbol	Genotype	Ontology Term	Ontology Annotation Term Name	Reference
MGI:105377	Adam19	Adam19^{tm1Asf}/Adam19^{tm1Asf}	MP:0000761	thin diaphragm muscle	(Kurohara et al., 2004)
MGI:109617	Barx2 Barx2 / Dmd (Myod)	Barx2^{tm1Rsd}/Barx2^{tm1Rsd} [Barx2^{tm1Rsd}/Barx2^{tm1Rs} Dmd^{mdx}/Dmd^{mdx}] [Barx2^{tm1Rsd}/Barx2^{tm1Rsd} Dmd^{mdx}/Y]	MP:0000761	thin diaphragm muscle	(Meech et al., 2012)
MGI:88294	Cacna1s	Cacna1s^{mdg}/Cacna1s^{mdg}	MP:0000761	thin diaphragm muscle	(PAI, 1965)
MGI:1888950	Cdkn1a	Cdkn1a^{tm1Led}/Cdkn1a^{tm1Led} Cdkn1c^{tm1Sje}/Cdkn1c^{+}	MP:0000761	thin diaphragm muscle	(Zhang et al., 1999)
MGI:88392	Chat	Chat$^{tm1.1Jrs}$/Chat$^{tm1.1Jrs}$	MP:0003924 MP:0000761	herniated diaphragm thin diaphragm muscle	(Misgeld et al., 2002)
MGI:87895	Chrng	Chrng$^{tm2(Chrne)Vwi}$/Chrng$^{tm2(Chrne)Vwi}$	MP:0000761	thin diaphragm muscle	(Pacifici et al., 2011)
	Chtop			Diaphragmatic hernia*	(Veenma, 2012. PhD Thesis)
MGI:94885	Des	Des^{tm1Cba}/Des^{tm1Cba}	MP:0000761	thin diaphragm muscle	(Li et al., 1996)
MGI:1329019	Dnase2a	Dnase2a^{tm1Aea}/Dnase2a^{tm1Aea}	MP:0003924 MP:0000761	herniated diaphragm thin diaphragm muscle	(Krieser et al., 2002)
MGI:2429765	Dock1	Dock1^{tm1Jfco}/Dock1^{tm1Jfco}	MP:0000761	thin diaphragm muscle	(Laurin et al., 2008)
MGI:1343461	Ecel1	Ecel1^{tm1Hiki}/Ecel1^{tm1Hiki}	MP:0000761	thin diaphragm muscle	(Nagata et al., 2010)
MGI:1891209	Efemp2	Efemp2$^{tm1.2Tynk}$/Efemp2$^{tm1.2Tynk}$	MP:0003924	herniated diaphragm	(Horiguchi et al., 2009)
MGI:95489	Fbn1	Fbn1^{tm2Rmz}/Fbn1^{tm2Rmz}	MP:0003924	herniated diaphragm	(Pereira et al., 1999)
MGI:1099809	Fgf10	Fgf10^{tm1Ska}/Fgf10^{tm1Ska}	MP:0003924	herniated diaphragm	(Min et al., 1998; Babiuk and Greer, 2002; Arkovitz et al., 2005; Sekine et al., 1999)
MGI:2150920	Fgfrl1	Fgfrl1^{tm1True}/Fgfrl1^{tm1True}	MP:0003924 MP:0000761	herniated diaphragm thin diaphragm muscle	(Baertschi et al., 2007; Catela et al., 2009)
MGI:2670972	Frem1	Frem1^{eyes2}/Frem1^{eyes2}	MP:0003924	herniated diaphragm	(Beck et al., 2013b; Beck et al., 2013a)
MGI:1917550	Fuz	Fuz^{b2b1273Clo}/Fuz^{b2b1273Clo}	MP:0003924	herniated diaphragm	
MGI:95664 MGI:3526856	Gata4	Gata4^{tm1Eno}/Gata4 $^{+}$ Gata4$^{tm1.2Wtp}$/Gata4 $^{+}$	MP:0003924	herniated diaphragm	(Jay et al., 2007)

MGI:95728 MGI:95729	Gli2 / Gli3	Gli2 (-/-) mice Gli3 (-/-) mice Gli2 (-/-) Gli3(+/-) double knockout mice		Diaphragmatic hernia*	(Motoyama et al., 1998; Kim et al., 2001)
MGI:96109	Hlx	$Hlx^{tm1Rph}/Hlx^{tm1Rph}$	MP:0003924	herniated diaphragm	(Hentsch et al., 1996)
MGI:96185 MGI:1857390	Hoxb4	$Hoxb4^{tm1Nrm}/Hoxb4^{tm1Nrm}$ $Hoxb4^{tm1Bay}/Hoxb4^{tm1Bay}$	MP:0003924	herniated diaphragm	(Ramirez-Solis et al., 1993; Manley et al., 2001)
MGI:1339973	Ilf3	$Ilf3^{tm1Pnk}/Ilf3^{tm1Pnk}$	MP:0000761	thin diaphragm muscle	(Shi et al., 2005)
	Kif7			Diaphragmatic hernia*	(Coles and Ackerman, 2013)
MGI:96795	Lmnb1	$Lmnb1^{tm1Yxz}/Lmnb1^{tm1Yxz}$ $[Lmnb1^{tm1Yxz}/Lmnb1^{tm1Yxz}\ Lmnb2^{tm1Yxz}/Lmnb2^{tm1Yxz}]$	MP:0000761	thin diaphragm muscle	(Kim et al., 2011)
MGI:5313524 MGI:3610732 MGI:2657016	Lox	$Lox^{b2b370.2Clo}/Lox^{b2b370.2Clo}$ $Lox^{tm1Soin}/Lox^{tm1Soin}$ $Lox^{tm1Ikh}/Lox^{tm1Ikh}$	MP:0003924 MP:0000761	herniated diaphragm thin diaphragm muscle	(Hornstra et al., 2003; Maki et al., 2005)
MGI:1857655 MGI:1858019	Met	$Met^{tm1Cbm}/Met^{tm1Cbm}$ $Met^{tm1Cpo}/Met^{tm1Cpo}$	MP:0003924 MP:0000761 MP:0011883	herniated diaphragm thin diaphragm muscle absent diaphragm	(Babiuk and Greer, 2002)
MGI:3046860 MGI:2386252	Mmp14 / Mmp2	$[Mmp14^{tm1Noda}/Mmp14^{tm1Noda}\ Mmp2^{tm1Ito}/Mmp2^{tm1Ito}]$	MP:0000761	thin diaphragm muscle	(Oh et al., 2004)
MGI:3655692 MGI:2148205	Msc (MyoR-) / Tcf21 ($capsulin^{LacZ}$)	$[Msc^{tm1Eno}/Msc^{tm1Eno}\ Tcf21^{tm2Eno}/Tcf21^{tm2Eno}]$	MP:0003924	herniated diaphragm	(Lu et al., 2002)
MGI:2182358 MGI:2182361 MGI:2182363	Mnx1	$Mnx1^{tm1Tmj}/Mnx1^{tm1Tmj}$ $Mnx1^{tm2Tmj}/Mnx1^{tm2Tmj}$ $Mnx1^{tm3Tmj}/Mnx1^{tm3Tmj}$	MP:0000761	thin diaphragm muscle	(Arber et al., 1999)
MGI:103581	Musk	$Musk^{tm1.1Vwi}/Musk^{tm2Vwi}$	MP:0000761	thin diaphragm muscle	(Chevessier et al., 2008)
MGI:1856328	Myod (Dmd)	Dmd^{mdx}/Dmd^{mdx} mdx:MyoD -/- ^{9th}			(Inanlou et al., 2003; Inanlou and Kablar, 2003)
MGI:97276	Myog	$Myog^{tm1Whk}/Myog^{tm1Whk}$	MP:0000761	thin diaphragm muscle	(Tseng et al., 2000; Hasty et al., 1993)
MGI:3579117	Nr2f2 ($Couptf\text{-}II^{Flox}$)	$[Nkx3\text{-}2^{tm1(cre)Tsa}/Nkx3\text{-}2^{+}\ Nr2f2^{tm2.1Tsa}/Nr2f2^{tm2.1Tsa}]$	MP:0003924	herniated diaphragm	(You et al., 2005)
MGI:96083	Nrg1	$Nrg1^{tm1Lwr}/Nrg1^{tm1Lwr}$	MP:0000761	thin diaphragm muscle	(Wolpowitz et al., 2000)
MGI:97487	Pax3	$Pax3^{tm1Mrc}/Pax3^{+}$	MP:0000761	thin diaphragm muscle	(Keller et al., 2004; Li et al., 1999)
MGI:97491	Pax7	$Pax7^{tm1Pgr}/Pax7^{tm1Pgr}$	MP:0000761	thin diaphragm muscle	(Seale et al., 2000)

	Pbx1			Diaphragm defects*	(Russell et al., 2012)
MGI:97530	Pdgfra	Pdgfra^{tm2Sor}/Pdgfra^{tm2Sor}	MP:0003924	herniated diaphragm	(Sun et al., 2000; Bleyl et al., 2007)
MGI:2158795 MGI:2158757	Ptprd / Ptprs	Ptprd^{tm1Yiw}/Ptprd^{tm1Yiw} Ptprs^{tm1Mtr}/Ptprs^{tm1Mtr}	MP:0000761	thin diaphragm muscle	(Uetani et al., 2006)
MGI:1857622 MGI:1857851	Rara / Rarb	[Rara^{tm1Ipc}/Rara^{tm1Ipc} Rarb^{tm1Mma}/Rarb^{tm1Mma}]	MP:0003924	herniated diaphragm	(Mendelsohn et al., 1994a; Lohnes et al., 1995)
MGI:1274781	Robo1			Diaphragmatic hernia*	(Xian et al., 2001)
MGI:102780	Six1	Six1^{tm1Mair}/Six1^{tm1Mair}	MP:0000761 MP:0011883	thin diaphragm muscle absent diaphragm	(Laclef et al., 2003b; Laclef et al., 2003a)
MGI:2679499 MGI:2662346 MGI:1315202	Slit3	Slit3$^{Gt(OST106158)Lex}$/Slit3$^{Gt(OST106158)Lex}$ Slit3^{tm1Dor}/Slit3^{tm1Dor} Slit3$^{tm1.1Dor}$/Slit3$^{tm1.1Dor}$	MP:0003924 MP:0000761	herniated diaphragm thin diaphragm muscle	(Liu et al., 2003; Yuan et al., 2003)
MGI:98331	Snap25	Snap25^{tm1Mcw}/Snap25^{tm1Mcw}	MP:0000761	thin diaphragm muscle	(Washbourne et al., 2002)
MGI:98369	Sox7	Sox7$^{tm1.1Dsco}$/Sox7^{+}	MP:0003924	herniated diaphragm	(Wat et al., 2012)
MGI:3587413	Zfpm2 (Fog2^{lil})	Zfpm2^{lil}/Zfpm2^{lil}	MP:0003924	herniated diaphragm	(Ackerman et al., 2005)
MGI:98968	Wt1	Wt1^{tm1Jae}/Wt1^{tm1Jae}	MP:0003924	herniated diaphragm	(Kreidberg et al., 1993; Clugston et al., 2006)

Table 1.2.1

Summarises the mouse models in which specific diaphragm abnormalities were observed; herniated diaphragm (CDH); thin diaphragm muscle; absent diaphragm. Information retrieved from MouseMine (http://www.mousemine.org/); Mouse Genome Informatics, MGI (http://www.informatics.jax.org/).

* From literature; Diaphragmatic defects or CDH observed.

Additional reference sources for Fuz, Lox: Modelling the genetic basis for human congenital heart disease in mice. (http://www.devbio.pitt.edu/research/mouse_muta.html).

ID	Gene Symbol	Genotype	Ontology Term	Ontology Annotation Term Name	Reference
MGI:87961	Agrn	[Agrn^{tm1Rwb}/Agrn^{tm1Rwb} Tg(Pax3-cre)1Joe/?]	MP:0002279	abnormal diaphragm morphology	(Harvey et al., 2007)
MGI:105058	Atp2a1	Atp2a1^{tm1Dhm}/Atp2a1^{tm1Dhm}	MP:0002279	abnormal diaphragm morphology	(Pan et al., 2003)
MGI:107437	Capn3	Capn3$^{Gt(OST141731)Lex}$/Capn3$^{Gt(OST141731)Lex}$	MP:0002279	abnormal diaphragm morphology	(Kramerova et al., 2004)
MGI:1923936	Clip3	Clip3$^{tm1.1Ics}$/Clip3$^{tm1.1Ics}$	MP:0002279	abnormal diaphragm morphology	(Couesnon et al., 2013)
MGI:88459	Col6a1	Col6a1^{tm1Gmb}/Col6a1^{+} Col6a1^{tm1Gmb}/Col6a1^{tm1Gmb}	MP:0002279	abnormal diaphragm morphology	(Bonaldo et al., 1998)
MGI:2183644 MGI:2183646	Ctbp1 / Ctbp2	[Ctbp1^{tm1Sor}/Ctbp1^{tm1Sor} Ctbp2$^{Gt(ROSA61)Sor}$/Ctbp2^{+}]	MP:0002279	abnormal diaphragm morphology	(Hildebrand and Soriano, 2002)
MGI:102720	Ednrb	Ednrb$^{s-15DttMb}$/Ednrb$^{s-15DttMb}$ Ednrb$^{s-1Acrg}$/Ednrb^{s-9ThW}	MP:0002279	abnormal diaphragm morphology	(Burgess et al., 2004)
MGI:95557	Flnc	Flnc^{tm1Lmk}/Flnc^{tm1Lmk}	MP:0002279	abnormal diaphragm morphology	(Dalkilic et al., 2006)
MGI:108088	Gab1	Gab1^{tm1Wbm}/Gab1^{tm1Wbm}	MP:0002279	abnormal diaphragm morphology	(Sachs et al., 2000)
MGI:1354951	Gne	[Gne^{tm1Sngi}/Gne^{tm1Sngi} Tg(ACTB-GNE*D176V)9Sngi/?]	MP:0002279	abnormal diaphragm morphology	(Malicdan et al., 2007)
MGI:2135593	Hadha	Hadha^{tm1Jib}/Hadha^{tm1Jib}	MP:0002279	abnormal diaphragm morphology	(Ibdah et al., 2001)
MGI:3576010	hpld	hpld/hpld	MP:0002279	abnormal diaphragm morphology	(Herron et al., 2002)
MGI:99954	Ighmbp2	Ighmbp2^{nmd-2J}/Ighmbp2^{nmd-2J}	MP:0002279	abnormal diaphragm morphology	(Grohmann et al., 2004)
MGI:102700	Itga7	Itga7^{tm1Burk}/Itga7^{tm1Burk} Itga7^{tm1Umr}/Itga7^{tm1Umr} [Itga7^{tm1Burk}/Itga7^{tm1Burk} Sspn^{tm1Kcam}/Sspn^{tm1Kcam}]	MP:0002279	abnormal diaphragm morphology	(Marshall et al., 2012; Mayer et al., 1997)
MGI:1342270	Large	Large^{myd}/Large^{myd} Large^{vls}/Large^{vls}	MP:0002279	abnormal diaphragm morphology	(Lee et al., 2005)
MGI:96794	Lmna	Lmna^{tm1Gbon}/Lmna^{tm1Gbon}	MP:0002279	abnormal diaphragm morphology	(Arimura et al., 2005)

MGI:1928394	Mtor	[Mtor$^{tm1.2Koz}$/Mtor$^{tm1.2Koz}$ Tg(ACTA1-cre)79Jme/?]	MP:0002279	abnormal diaphragm morphology	(Risson et al., 2009)
MGI:2179432	Mycbp2	Mycbp2$^{tm1.1Adia}$/Mycbp2$^{tm1.1Adia}$	MP:0002279	abnormal diaphragm morphology	(Bloom et al., 2007)
MGI:1100535	Myt1	Myt1$^{tm1.1Ggu}$/Myt1$^{tm1.1Ggu}$	MP:0002279	abnormal diaphragm morphology	(Wang et al., 2007)
MGI:2444155	Nmnat2	Nmnat2$^{Tn(sb-Tyr)2172.P9KK4BOve}$/Nmnat2$^{Tn(sb-Tyr)2172.P9KK4BOve}$	MP:0002279	abnormal diaphragm morphology	(Hicks et al., 2012)
MGI:5427579 MGI:2156086	Pik3ca / Pten	[Pik3ca$^{tm1.1Waph}$/Pik3ca$^{tm1.1Waph}$ Pten^{tm1Hwu}/Pten^{tm1Hwu}]	MP:0002279	abnormal diaphragm morphology	(Kinross et al., 2012)
MGI:96522	Rbpj	[Pax3$^{tm1(cre)Joe}$/Pax3^{+} Rbpj^{tm1Hon}/Rbpj^{tm1Hon}]	MP:0002279	abnormal diaphragm morphology	(Vasyutina et al., 2007)
MGI:99659	Ryr1	Ryr1$^{tm1.1Dhm}$/Ryr1$^{tm1.1Dhm}$	MP:0002279	abnormal diaphragm morphology	(Zvaritch et al., 2007)
MGI:1346524	Sgcg	Sgcg^{tm1Mcn}/Sgcg^{tm1Mcn} Sgcg^{tm1Oza}/Sgcg^{tm1Oza}	MP:0002279	abnormal diaphragm morphology	(Hack et al., 1998; Sasaoka et al., 2003)
MGI:106658	Srf	[Srf$^{tm2.1Nor}$/Srf$^{tm2.1Nor}$ Tg(Myog-cre)1Eno/?]	MP:0002279	abnormal diaphragm morphology	(Li et al., 2005)
MGI:98725	Tgfb1	Tgfb1^{tm1Doe}/Tgfb1^{tm1Doe}	MP:0002279	abnormal diaphragm morphology	(Shull et al., 1992)

Table 1.2.2.

Summarises the additional mouse models in which abnormal diaphragm morphology was observed. Information from MouseMine (http://www.mousemine.org/); Mouse Genome Informatics, MGI (http://www.informatics.jax.org/); and from recent literature.

1.2.6 Current Hypotheses on the Developmental Origins of CDH.

Over recent years three main concepts emerge; the retinoid hypothesis, the mesenchymal-hit hypothesis, and the dual-hit hypothesis. These three hypotheses are supported by growing evidence from dietary, teratogenic (nitrofen, BMS493), and genetic animal models, and from identification of genetic abnormalities in human CDH.

1.2.6.1 The Retinoid Signalling Pathway and the Retinoid Hypothesis

Vitamin A is essential for various aspects of early embryonic development, as demonstrated by the spectrum of abnormalities observed in vitamin A deficient (VAD) animal models (Wilson et al., 1953). The observation that CDH is associated with VAD models, and with teratogen-induced models such as nitrofen, which disrupt the retinoic acid (RA), or retinoid signalling pathway, provides strong evidence for a link with diaphragm development and this pathway (Thebaud et al., 1999; Babiuk et al., 2004; Oshiro et al., 2005; Nakazawa et al., 2007b; Nakazawa et al., 2007a; Noble et al., 2007; Clugston et al., 2010). More recently, genetic models for various genes known to be involved in the RA pathway have provided further evidence of a link to CDH. These models include the FOG2 ENU mutant, the COUP-TFII knockout model, the heterozygous $GATA4^{+/\Delta ex2}$ model, and the retinoic acid receptor (RAR) double knockout models (see table 1). However, it is of interest that the Bochdalek-type hernias, which account for the vast majority of human CDH cases, have not been observed in the FOG2 or GATA4 mutants. The retinoic acid pathway has been described in a number of publications, including (Clagett-Dame and DeLuca, 2002; Montedonico et al., 2008; Niederreither and Dolle, 2008; Klaassens et al., 2009; Theodosiou et al., 2010; Goumy et al., 2010), and an overview is presented in Figure 1.2.4.

FOG2 (also known as ZFPM2) is a zinc finger transcription cofactor which modulates the activity of GATA transcription factors, in particular GATA4, and is located on 8q23, a region associated with CDH in humans (OMIM 610187, DIH3). Using mice treated with N-ethyl-N-nitrosourea (ENU), Ackerman *et al* identified a FOG2 mutation generating a truncated protein and causing pulmonary hypoplasia and abnormal diaphragmatic development (Ackerman et al., 2005). Furthermore, the authors identified a *de novo* mutation in 1 of 30 deceased children with diaphragm defects, in which severe bilateral pulmonary hypoplasia and an abnormally muscularised diaphragm were observed. Bleyl *et al* later identified novel sequence alterations in 2 of 96 patients with isolated CDH, however were unable to

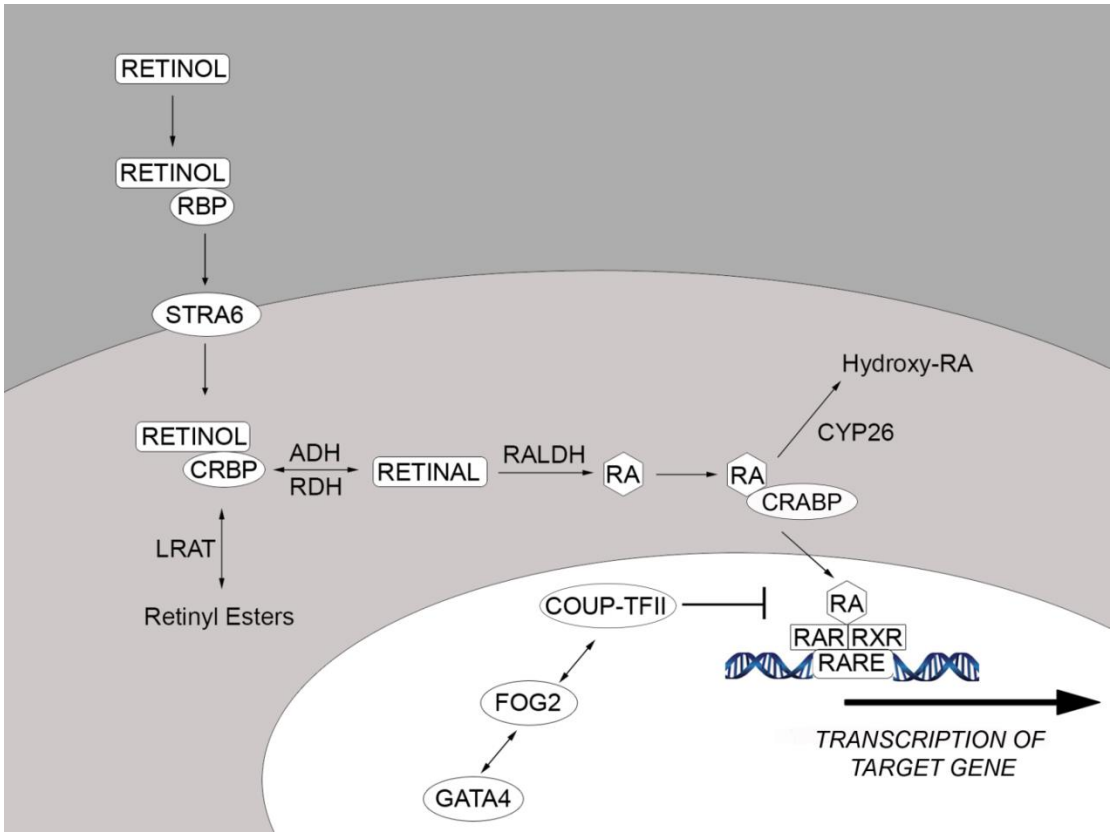

Figure 1.2.4. An overview of the retinoic acid pathway.

Retinol is transported to target cells in a complex with RBP, with transfer into the cell occurring by the cell surface receptor STRA6. Within the target cell, retinol is bound to CRBP, and can be converted to retinal by ADH (in a reversible reaction), or to retinyl esters by LRAT. Retinal is oxidised to retinoic acid (RA) by RALDH in an irreversible reaction, whereby RA can complex with CRABP which enables transport to the nucleus. Within the nucleus RA forms a complex with RAR/RXR heterodimers, which in turn bind to a short DNA sequence, the retinoic acid response element (RARE) thereby activating transcription of the target gene. COUP-TFII can inhibit the heterodimerisation of RAR/RXR, thus inhibiting gene transcription. FOG2 interacts with both COUP-TFII and GATA4, thereby modulating the activity of GATA4 transcription factors. Excess cellular RA can be presented to CYP26 enzymes for degradation to Hydroxy RA. Nitrofen is believed to exert its effects through inhibition of RALDH activity.

Abbreviations: RBP (retinol binding proteins). STRA6 (stimulated by retinoic acid 6). CRBP (cellular retinol binding proteins). ADH (alcohol dehydrogenases). RDH (retinol dehydrogenases). RALDH (retinaldehyde dehydrogenases). RA (retinoic acid). CRABP (cellular retinoic acid binding proteins). RAR (retinoic acid receptors). RXR (retinoid X receptors). RARE (retinoic acid response element). LRAT (lecithin:retinol acetyltransferase). CYP26 (cytochrome P450 family 26).

confidently determine the variants as causal mutations due to the lack of parental samples (Bleyl et al., 2007).

COUP-TFII is a transcription factor and orphan nuclear receptor which can interact with FOG2. COUP-TFII can inhibit gene transcription by preventing heterodimerisation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), as well as modulate the transcriptional activity of GATA proteins, particularly GATA4. You *et al* generated tissue-specific null mutants of COUP-TFII which displayed the common Bochdalek type of CDH. Targeted ablation of COUP-TFII in the foregut mesenchyme, including the posthepatic mesenchymal plate (PHMP), which is also referred to as the PPF, resulted in malformation of the diaphragm and the failure of the PHMP to attach to the body wall (You et al., 2005). The location of COUP-TFII also known as NR2F2, on chromosome 15q26, a region recurrently associated with CDH patients makes this gene an interesting candidate for human CDH (Klaassens et al., 2005; Slavotinek et al., 2006). However, the study of Scott *et al* was unable to identify COUP-TFII mutations in 73 CDH samples (Scott et al., 2007). In another study, Slavotinek *et al* studied 6 candidate genes in the 15q26 critical region in over 100 CDH patients (Slavotinek et al., 2006). However, although mis-sense changes were identified, none of these alterations could be assigned as definitively causal for CDH. This data may be suggestive of chromosome deletions at 15q26 being a contiguous gene deletion syndrome, or may be due to the suggested multi-factorial etiology of CDH.

GATA4 is a zinc finger transcription factor known to interact with FOG2 (Crispino et al., 2001) and involved in regulating gene expression (Molkentin, 2000). GATA4 null mutant mice are embryonic lethal due to the essential role of this gene in heart development (Kuo et al., 1997; Molkentin et al., 1997). However, Jay *et al* generated a mouse model with a heterozygous deletion in exon 2 of GATA4 which displayed CDH and primary lung abnormalities. GATA4 is located in the critical region of the 8p23.1 deletion syndrome which shows a strong association with CDH (Wat et al., 2009) as well as heart defects. However, in humans GATA4 mutations are typically associated with heart abnormalities and not with CDH (Garg et al., 2003; Okubo et al., 2004; Tomita-Mitchell et al., 2007; Rajagopal et al., 2007; Reamon-Buettner and Borlak, 2005; Reamon-Buettner et al., 2007).

Several studies have not observed defects in embryological development for individual RAR knockout models (Li et al., 1993; Mendelsohn et al., 1994b), indicating a degree of overlapping function and compensation by the α, β, and γ forms of receptor. However, generation of compound null RAR mutants were shown to have a spectrum of VAD-like

defects including diaphragm defects (Lohnes et al., 1994; Mendelsohn et al., 1994a; Lohnes et al., 1995), confirming the importance of these receptors for early development in a number of systems. It has recently been proposed that the unique expression pattern of RARα in the PPF may be of particular importance for diaphragm development (Clugston et al., 2010).

In humans, mutations in STRA6 (stimulated by retinoic acid 6), a retinol binding protein (RBP) receptor, have recently been linked to a spectrum of defects including diaphragmatic and pulmonary defects known collectively as Matthew Wood syndrome (Golzio et al., 2007; Pasutto et al., 2007; Chassaing et al., 2009), providing evidence for the involvement of this pathway in human CDH.

Collectively there is an increasing amount of evidence which supports the hypothesis that abnormal retinoid signalling plays an important role in the development of CDH. Further investigation and the generation of additional novel models which are targeted to specific components of the retinoid signalling pathway will increase our understanding of the pathogenesis of CDH and of the role that retinoid signalling and the downstream targets of this pathway play in diaphragm development. However, given the spectrum of defects seen in humans with STRA6 mutations, as well as the evidence for the RSP having roles in various aspects of embryological development (reviewed in (Niederreither and Dolle, 2008)), it remains to be determined whether defects at the higher levels of this pathway are responsible for isolated CDH.

1.2.6.2 PPF development and the Mesenchymal Hit Hypothesis

There is a body of evidence from animal models that CDH originates from a malformation of the PPFs, which are a key structure in the embryogenesis of the diaphragm being the target for muscle precursor cells and for the phrenic nerve. It has been hypothesised that abnormal PPF development may underlie Bochdalek CDH (Allan and Greer, 1997; Clugston and Greer, 2007; Clugston et al., 2009). A number of histological studies have compared this structure in both normal and abnormal situations, and 3D reconstructions have also been generated by some groups (Clugston et al., 2006; Clugston et al., 2009; Ackerman and Greer, 2007). Through the use of histological investigations, Clugston *et al* have demonstrated that teratogen-induced, dietary and genetic models of CDH appear to share a common mechanism of pathogenesis originating from defects in the PPF (Clugston et al., 2006). More recently, the same group investigated the timeframe of PPF formation in rat embryos and

human embryos, identifying a critical period of normal diaphragm development at 4-6 weeks gestation. Importantly, this time period is earlier in gestation than conventionally thought and occurs before the typical time-point of pregnancy determination. Through additional investigations using nitrofen-exposed NIH 3T3 cells *in vitro*, it is proposed that impaired cell proliferation, and not increased apoptosis, contributes to abnormal diaphragm development in the nitrofen model of CDH (Clugston et al., 2009).

Expression analysis in the diaphragm has revealed interesting results supporting the mesenchymal hit hypothesis. Analysis of wild-type mouse embryos demonstrated co-expression of GATA4 and FOG2 in mesenchymal cells of the developing diaphragm, lungs and heart (Jay et al., 2007). Double immuno-labelling for COUP-TFII and WT1 has shown that these proteins co-localise within the non-muscular mesenchymal cells of the developing PPF (Clugston et al., 2006). These co-expression data indicate that the COUP-TFII, FOG2, GATA4 and WT1 models provide support for the mesenchymal hit hypothesis.

Clugston *et al* found that RALDH2 is the only retinal dehydrogenase isoform expressed in the developing PPF. Examination of retinoid receptor expression in the PPF for the α, β, and γ isoforms of both the RARs and RXRs revealed positive expression for only RARα, RARγ, and RXRα. These receptors were shown to be strongly expressed within the non-muscular mesenchymal cells (WT1 positive), with weak expression in the muscle precursor cells (PAX3 positive). Interestingly, whilst the RARγ and RXRα expression was relatively uniform and widespread, the expression pattern of RARα was largely restricted to the caudal region of the PPF, corresponding to the affected region of the PPF in nitrofen-exposed embryos. It is therefore now proposed that RARα signalling in the developing PPF may be of particular importance for normal diaphragm development (Clugston et al., 2010). The finding that the PPF is a centre for retinoid signalling further implicates this pathway and provides a potential link to defective retinoid signalling causing abnormal PPF development and thus CDH by affecting the mesenchymal component of this tissue early in diaphragm development (Clugston et al., 2010).

Additional research has shown that the 15q26 region contains a cluster of genes that are expressed in the developing diaphragm in rodents, supporting the association of CDH with deletions of 15q26. Further examination of protein expression within the developing diaphragm has shown that genes strongly associated with CDH (COUP-TFII, FOG2, GATA4, and WT1) are expressed only in the non-muscular mesenchymal component of the diaphragm, supporting the mesenchymal hit hypothesis, and well as demonstrating that

these factors are all co-expressed in the same cells. This is suggestive of different genetic causes leading to a common defect in the mesenchymal cells of the PPF which causes CDH, supporting the theory that unique genetic defects affecting a common pathway can lead to CDH (Clugston et al., 2008).

Given the apparent defective development of the PPF in multiple animal models of Bochdalek type CDH, as well as in archived human embryo specimens (Clugston et al., 2009) further investigation of the role that this structure plays, and in particular the role of the non-muscular mesenchymal cells in the pathogenesis of isolated CDH is needed. It will also provide an improved understanding of the embryogenesis of the diaphragm, and the influence that defects in the PPFs have on the development of CDH in humans. It is essential for us to understand the genetic factors involved in normal PPF development and how genetic defects can affect the PPF and further development of the diaphragm.

1.2.6.3 Diaphragm and Lungs – the Dual Hit Hypothesis

Keijzer *et al* demonstrated using lung explants that nitrofen interferes with early lung development before and separate from aberrant diaphragm development. The authors postulate the dual-hit hypothesis to explain pulmonary hypoplasia in CDH by two mechanisms, the first affecting both lungs before diaphragm development and a second affecting the ipsilateral lung after defective diaphragm development (Keijzer et al., 2000). Nitrofen is however damaging at high doses to a number of developing organs which are dependent on retinoid signalling. It is possible that the diaphragm may be particularly sensitive to disruption of retinoid signalling in the case of CDH.

The FOG2 ENU mouse model implicates the role of this gene in the pathogenesis of isolated CDH, and its necessity for pulmonary development validates the hypothesis that neonates with CDH may also have primary pulmonary developmental abnormalities. Furthermore, expression analysis of Gata4 and Fog2 in wild-type mouse embryos has demonstrated co-expression in the mesenchymal cells of the developing diaphragm, lungs and heart (Jay et al., 2007).

Recently, it has also been shown that WT1 is specifically expressed in the embryonic mesothelium. WT1 expressing cells were abundant from the origin of the lung buds to postnatal stages contributing significantly to pulmonary endothelial and smooth muscle cells, bronchial musculature, tracheal and bronchial cartilage. Thus, Wt1cre-YFP

mesenchymal cells show the very same differentiation potential than the splanchnopleural mesenchyme surrounding the lung buds. We have also found defects in pulmonary development in Wt1-/- embryos, which showed abnormally fused lung lobes, round-shaped and reduced pleural cavities and diaphragmatic hernia. Our results suggest a novel role for the embryonic mesothelium-derived cells in lung morphogenesis and involve the Wilms' tumor suppressor gene in the development of this organ (Cano et al., 2013)

The overlapping function of certain genes and pathways in both diaphragm and lung development supports the dual-hit model, however, exactly how defects in retinoid signalling and mesenchymal cell function may contribute to cases of isolated CDH remains to be determined.

1.2.7 The Contribution of Environmental Factors

It has become increasingly evident that genetic factors play an important role in the aetiology of CDH (Slavotinek, 2007; Bielinska et al., 2007). However, it is still uncertain whether complex genetic factors are the sole cause of CDH. The evidence from teratogen- and dietary-induced CDH animal models is suggestive of environmental and/or nutritional factors also playing a role (reviewed in (Beurskens et al., 2009)). A recent study which investigated the Vitamin A status of mothers and their newborns in association with CDH found a strong association with low retinol and RBP independent of maternal retinol levels (Beurskens et al., 2010). These findings are more consistent with there being an abnormality in embryological retinoid homeostasis in the foetus, rather than maternal vitamin A deficiency being a risk factor. More research in this area is certainly warranted since maternal malnutrition and lifestyle factors are easily modified, which may potentially reduce the risk of CDH and other congenital malformations.

The variable penetrance of CDH in teratogen-induced and genetic models of different strains (Jay et al., 2007; Beck et al., 2013a) is suggestive of a genetic background which confers a susceptibility to the development of CDH. The same is true in humans. The observation that CDH does not display full penetrance for genetic disorders or genomic loci adds further weight to as yet unidentified regulatory factors or environmental influences also playing an important role.

Chapter 2

Research Objectives

Chapter 2. Research Objectives

We propose that a significant proportion of congenital malformations are caused by genomic imbalances, and that a number of these CNVs are undetectable by prenatal karyotype. We hypothesise that the application of high resolution genome-wide array CGH for prenatal diagnosis will reveal novel submicroscopic loci which are important for foetal development. Furthermore, the higher resolution provided by array CGH in comparison to conventional karyotyping, and the faster result time, will improve the standard of analysis offered for prenatal genetic diagnosis. In **Chapter 3** we present the results of our study into the clinical utility of chromosomal microarray analysis for prenatal diagnosis in referrals with abnormal ultrasound findings, and our approach to the reporting of VOUS and risk loci during pregnancy.

One developmental disorder for which the genetic basis in humans remains poorly understood is congenital diaphragmatic hernia (CDH). While much information has been gained from the study of model organisms, the genetic causes of isolated CDH remain largely unknown. Currently those foetuses with moderate-severe isolated CDH are the focus of foetal therapy. Since a subset of these patients may have a poor prognosis due to an underlying genetic abnormality, it is important to make a diagnosis where possible allowing better informed decisions for possible therapies. Moreover, improving our understanding of the pathogenesis of CDH will reveal novel targets and pathways for future foetal therapies.

We propose that genetic causes underlie a significant proportion of isolated CDH cases and that chromosomal microarray analysis will identify genomic imbalances revealing candidate loci and genes associated with isolated CDH. In **Chapter 4** we present the results of our retrospective study of isolated CDH using a custom design array. In the second part of **Chapter 4** we present the results from our prospective prenatal study of isolated CDH foetuses using a genome-wide array.

Exome sequencing allows for screening of the entire coding portion of the genome and has the potential to identify causal mutations involved in development of CDH. In **Chapter 5** we present our findings from exome sequencing in cases of familial CDH.

In **Chapter 6** we present the results of an exploratory study using RNA-Sequencing to investigate gene expression patterns in amniotic fluid cells of isolated CDH foetuses. The dysregulated genes and pathways identified can potentially be used to identify subtypes of

CDH based upon the gene expression profiles which may correlate with, for example, severity of disease or response to treatment.

Finally, in **Chapter 7** we discuss the implications of our findings and consider future perspectives.

Chapter 3

Prenatal Chromosomal Microarray Analysis

Chapter 3. Prenatal Chromosomal Microarray Analysis.

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A prospective study of the clinical utility of prenatal chromosomal microarray analysis in foetuses with ultrasound abnormalities and an exploration of a framework for reporting unclassified variants and risk factors.

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Abstract

Purpose: To evaluate the clinical utility of chromosomal microarrays for prenatal diagnosis by a prospective study of foetuses with abnormalities detected on ultrasound.

Methods: Patients referred for prenatal diagnosis due to ultrasound anomalies underwent analysis by array CGH as the first-tier diagnostic test.

Results: A total of 383 prenatal samples underwent analysis by array CGH. Array analysis revealed causal imbalances in a total of 9.6% of patients (n=37). Submicroscopic copy number variations (CNVs) were detected in 2.6% of patients (n=10/37), and arrays added valuable information over conventional karyotyping in 3.9% of patients (n=15/37). We

highlight a novel advantage of arrays; a 500kb paternal insertional translocation is the likely driver of a *de novo* unbalanced translocation, thus improving recurrence risk calculation in this family. Variants of uncertain significance were revealed in 1.6% of patients (n=6/383).

Conclusion: We demonstrate the added value of chromosomal microarrays for prenatal diagnosis in the presence of ultrasound anomalies. We advocate reporting back only CNVs with known pathogenic significance. Although this approach might be considered opposite to the ideal of full reproductive autonomy of the parents, we argue why providing all information to parents may result in a false sense of autonomy.

Introduction

Conventional karyotyping has been considered the gold standard for routine prenatal genetic diagnosis for many decades now, allowing for microscopic visualisation and inspection of chromosomes and thus detection of numerical and structural chromosomal rearrangements. The main limitations are the resolution achieved by G-banding, which is limited to 5-10Mb at best, and the requirement for cultured cells, needing a minimum of 8-10 days. The introduction of targeted methods of analysis such as fluorescence *in situ* hybridisation (FISH) and multiplex ligation-dependent probe amplification (MLPA) overcome the time constraints and resolution limitations inherent to karyotyping, but do not provide a genome-wide analysis (Goumy et al., 2010). More recently, molecular karyotyping using genomic microarrays has reached mainstream use in the postnatal diagnostic setting providing a genome-wide screen for genomic imbalances at a far superior resolution to karyotyping (Miller et al., 2010). A number of studies have demonstrated the feasibility of prenatal diagnosis by genomic arrays using a variety of platforms (Coppinger et al., 2009; Faas et al., 2010; Srebniak et al., 2011; Breman et al., 2012; Shaffer et al., 2012), but challenges remain in applying high resolution genomic arrays to prenatal diagnosis (Vetro et al., 2012; Wapner et al., 2012a; Breman et al., 2012). One of the major ethical issues often raised is how to deal with variants of uncertain significance (VOUS) or risk loci, the detection of which leads to additional challenges for genetic counselling of parents. Furthermore, array analysis may reveal an imbalance for known 'risk loci' where the future penetrance is uncertain or may be associated with variable expression. The penetrance risks for a number of recurrent CNVs have been estimated based upon the frequencies in patients and controls (Kaminsky et al., 2011; Cooper et al., 2011; Rosenfeld et al., 2013). However, while it is possible to calculate a population-based risk, it is impossible in the prenatal setting to predict the phenotypic outcome in the child.

Here, we present results of a prospective study on the routine use of array CGH for foetuses with congenital abnormalities detected prenatally by ultrasound from 2 Belgian genetics centres. We also discuss our approaches to the interpretation and reporting of genomic array results in the prenatal setting.

Methods

Referral Criteria and Time Period

Samples were received over a 3-year period at the Centre for Human Genetics (CME), KU Leuven, UZ Leuven and the Centre for Medical Genetics Ghent (CMGG), Ghent University Hospital (herein referred to as KUL and UG, respectively). For both centres, criteria for inclusion were either (i) multiple abnormalities, or (ii) isolated abnormality observed on ultrasound for which invasive testing and genetic analysis is advised. Ethical approval was granted for this study in both centres.

Sample Types

Chorionic Villi Samples (CVS), Amniotic Fluid (AF) samples and foetal blood samples were received. In all cases backup cultures were initiated for further DNA requirements and for conventional karyotype. When available, genomic DNA was immediately isolated from fresh material (including all foetal blood samples). Where required, DNA was isolated from cultured cells (AF and CVS only). Villi from CVS were separated from maternal tissue under a microscope to minimise maternal cell contamination (MCC). Two to four villi were provided for DNA extraction. Between 2ml and 13ml (mean=7; median=8) of AF was provided for DNA extraction. AF samples were centrifuged and cell pellets washed twice in PBS. Genomic DNA was extracted by both centres using the DNA mini kit (QIAGEN Benelux B.V. – Belgium, Venlo, Netherlands) following the manufacturers recommendations. For CVS, incubation at 56°C with proteinase K and tissue lysis buffer (Buffer ATL) was performed for at least 1 hour for efficient digestion and lysis of the complete sample. For AF, incubation at 56°C with proteinase K and lysis buffer (Buffer AL) was performed for 10 minutes. Amicon YM-30 Spin Columns (Merck Millipore S.A./N.V., Overijse, Belgium) were used for concentrating DNA samples (KUL). Total DNA yields for the first 100 samples including uncultured CVS and AF ranged from 1µg to 6µg, and from 100ng to 2µg (mean=800ng), respectively. MCC was assessed on DNA for all CVS by using the DNA Purity Assay (Multiplicom, Niel, Belgium), or the PowerPlex system (Promega Benelux B.V., Leiden,

Netherlands), both of which utilise polymorphic STR marker analysis. MCC was assessed on DNA for heavily blood-stained AF only.

Genomic Array Platforms

KUL applied the CytoSure Syndrome Plus 105K array and CytoSure Syndrome Plus 180K array (Oxford Gene Technology, Oxford, UK). This platform has genome-wide coverage with enrichment of target regions. Details of the array designs are available from Oxford Gene Technology (http://www.ogt.co.uk/) or the authors. UG applied the Agilent 60K platform (AMADID 21924) or the Agilent 180K platform (AMADID 27676) (Agilent Technologies S.A./N.V., Diegem, Belgium) which is the International Standards for Cytogenomic Arrays (ISCA) Consortium design, supplemented with selected genes / loci of interest. Details of the array designs are available from Agilent Technologies or the authors.

Array CGH Analysis and Interpretation

Genomic DNA was labelled for 4hrs using the CytoSure Labelling Kit (Oxford Gene Technology), with no enzyme digestion. Hybridisation was performed from 24 – 60 hours, in a rotator oven (SciGene, CA, USA) at 65'C. Washing of arrays was performed using Agilent wash solutions manually or with a Little Dipper Microarray Processor (SciGene), and dried using acetonitrile. Arrays were scanned using an Agilent microarray scanner at 2µm resolution, followed by calculation of signal intensities using Feature Extraction software (Agilent Technologies). Visualisations of results and data analysis were performed using the CytoSure Interpret Software (Oxford Gene Technology) and the CBS algorithm. The calling thresholds were deviation of a circular binary segmentation (CBS) segment from zero log ratio of; +0.36 for duplications and -0.72 for deletions, and containing ≥5 oligonucleotide probes. All samples were hybridized twice in dye swap experiments, labelled with Cy5 and Cy3 and hybridized versus Cy3 and Cy5 labelled reference DNA, respectively. The dye swap increases the sensitivity which, in turn, allows a more accurate detection of smaller imbalances, refinement of the breakpoint and mosaicism. No major discrepancies are observed between hybridisations. Results were then classified with CytoSure Interpret Software (Oxford Gene Technology) and with BENCH software (Cartagenia N.V., Leuven, Belgium). Quality control metrics are monitored with CytoSure Interpret software (Oxford Gene Technology). For UG, arrays were hybridised and analysed as previously described (Buysse et al., 2009). Genomic coordinates are based upon build hg18.

Pre-test Counselling and Informed Consent

Following detection of an abnormality on ultrasound investigation, array analysis was offered as the first-tier test with additional multidisciplinary counselling and informed consent at a tertiary centre. Traditional chromosome analysis by conventional karyotyping was not routinely performed. Rapid aneuploidy detection (RAD) was performed by FISH or qf-PCR for all patients prior to array analysis to timely exclude common autosomal and sex chromosome aneuploidies, as well as to exclude triploidy. Parental samples may be required for full interpretation of array results, and were provided along with the prenatal sample where possible. Parental samples were only analysed after detection of a prenatal imbalance, and only for those regions of interest. Patients agree during the informed consent that all causal and clinically significant imbalances, but not all variants of uncertain significance (VOUS) or (inherited) likely benign CNVs are reported (routinely). This data is retained by the laboratory and available on request. VOUS were defined as CNVs which have not been described before in the literature as pathogenic nor contain genes known to cause haploinsufficiency syndromes, but are likely deleterious, based on the functional evidence known about the genes in the CNVs. The published recommendations and guidelines for classification and interpretation of CNVs for constitutional postnatal genetic diagnosis, including VOUS, form the basis for our CNV classification in the prenatal setting also (Vetro et al., 2012; Kearney et al., 2011; Vermeesch et al., 2012; de Leeuw et al., 2012). CNVs without genes or known to be common in the population are considered benign. A list of common polymorphic CNVs is curated by the laboratory based upon in-house data and prior experience, supported by additional evidence from the database of genomic variants (DGV), DECIPHER and ISCA databases (Table S2, online supplementary information). A clinical geneticist assists the laboratory in the final decision of CNV classification and advises which imbalances to report to the referring clinician and patient in challenging cases.

Confirmation of findings

All pathogenic CNVs were confirmed by conventional karyotype, FISH analysis, MLPA, and / or qf-PCR.

Results

A combined total of 403 prenatal samples underwent analysis by array CGH. Of these 403 samples received, an interpretable result was obtained in 383 cases. For 20 samples the array result could not be interpreted due to either maternal cell contamination (MCC) (n=4),

or a poor quality array result with a derivative log ratio (DLR) value beyond an acceptable range of 0.3 (n=16). Of total samples received 65% (n=262) were AF, 21% (n=85) were CVS and 14% (n=56) were foetal blood samples. Only common polymorphic and likely benign CNVs were observed for 289/383 (75.5%) of patients and a normal array result was reported. In the remaining 24.5% of patients (n=94), rare and/or likely pathogenic CNVs were detected, which are further detailed below. For 51/383 (13.3%) of patients, the findings were classified as rare inherited and likely benign CNVs following parental array analysis. The information about those variants was deemed not of clear 'clinical relevance' based upon current knowledge and hence was not reported back to parents. However, it cannot be excluded that such inherited variants could be pathogenic in the offspring due to incomplete penetrance or due to variable expressivity (for the rationale, see discussion). Array analysis revealed causal imbalances in 37/383 (9.6%) of patients, 10 of which (2.6%) were submicroscopic. This means around ¼ of all causal imbalances are submicroscopic and thus undetectable by conventional karyotype alone. In 3.1% (n=12) of patients with abnormal array results (30% of causal imbalances) concordance with the subsequent abnormal karyotype was observed. For 1.6% of patients (n=6), VOUS were detected and the decision of whether to report these VOUS or not was determined on a case-by-case basis, for which the rationale is described further below.

The workflow shown in Figure 1 summarises our results, with further details of causal imbalances revealed by array analysis, as well as incidental findings of clinical significance and variants of uncertain significance (VOUS) provided in Table S1 (online supplementary information). Notable examples are discussed in more detail below.

Submicroscopic CNVs

A 6Mb deletion of 1p36 was observed in foetus KUL 2 with an Ebstein malformation and cerebral ventriculomegaly. Interestingly, 1p36 deletions have occasionally been associated with this rare malformation and this case adds further evidence of a link between this rare heart abnormality and haploinsufficiency of one or more genes at 1p36 (Witters et al., 2002; Digilio et al., 2011). In foetus KUL 4, referred due to semi-lobar holoprosencephaly, a novel 2 Mb *de novo* deletion of 10q24.31-q24.32 was observed. Within the deleted region several genes are located. However, we consider *SUFU* haploinsufficiency as the most likely cause for this phenotype since SUFU is a negative regulator of hedgehog signalling (Humke et al., 2010; Kim et al., 2011). Genetic variants in this signalling pathway, including in *SHH* (OMIM#142945) and *GLI2* (OMIM#610829), are linked to holoprosencephaly in humans.

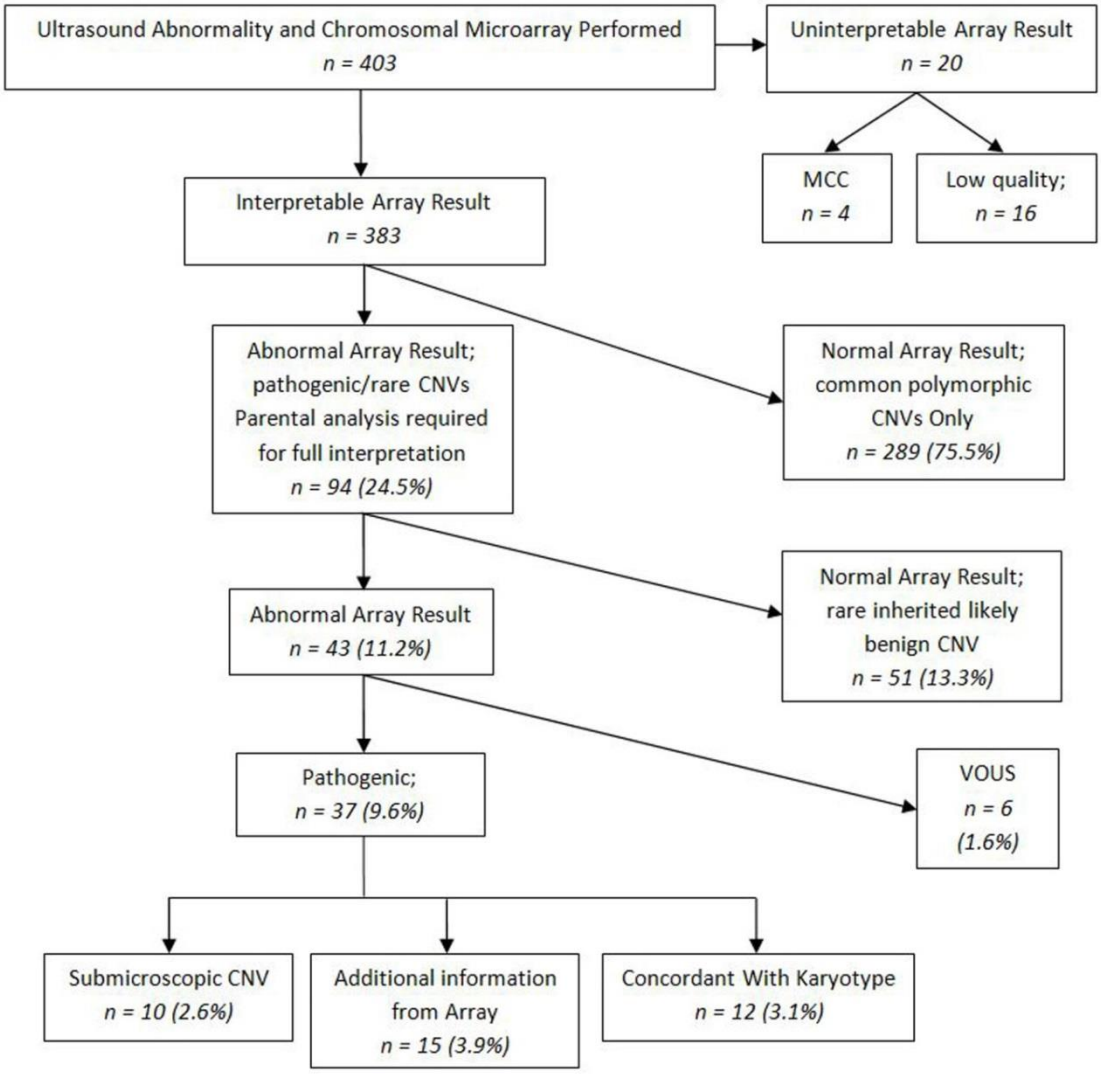

Figure 1. Workflow of chromosomal microarray results.

Arrays Reveal Additional Information above Karyotype

In 15/383 (3.9%) of foetuses, array analysis revealed additional information above karyotyping alone. These are listed in Table S1 (online supplementary information), and include inverted deletion duplications (inv del dups), unbalanced translocations and marker chromosomes. One case of interest was foetus KUL 17 for which deletion of 21q22.3, amplification (i.e. 4 or more copies) of 21q22.2-q22.3, and duplication of 21q21.3q22.2 were observed on both uncultured and cultured CVS material (Figure 2). The duplicated region displays a reduced dynamic range (i.e. reduced deviation of Log2 signal intensity ratios from zero, as opposed to the theoretical deviation of +0.58, and the experimentally observed deviations of +0.45 to +0.55), suggestive of mosaicism. MCC was excluded by STR marker analysis of foetal and parental material. Confined placental mosaicism could not be fully

excluded since no AF, foetal blood or foetal skin fibroblast sample was obtained. The initial rapid aneuploidy testing by FISH analysis with a centromeric probe for chromosome 21 revealed 3 signals, and the subsequent karyotype revealed 1 normal and 1 abnormal (possible ring) chromosome 21. This finding represents a mosaicism with different cell lines which have undergone different numbers of breakage-fusion-bridge cycles (Voet et al., 2011), which has, to our knowledge, not been described previously in a constitutional disorder.

In foetus KUL 15 referred due to cardiac anomalies a 17.5 Mb duplication of 22q12.3-q13.33 was detected (Figure 3A). Subsequent prenatal karyotype revealed translocation of the duplicated 22q fragment to the 22p arm (Figure 3C). Parental karyotypes were both normal. However, parental array analysis revealed a 500 kb duplication of 22q12.3 in the paternal sample (Figure 3B). This 500kb region overlaps with the proximal region of the terminal 22q duplication present in the foetus. We hypothesised this 500kb region to be an insertional translocation within the 22p microsatellite, which was confirmed by subsequent FISH analysis of the paternal sample (probe RP11-413J08) (Figure 3D). The translocation was thus most likely due to a crossing over in the insertional translocation in the father. This may be the first direct support that a submicroscopic insertional translocation can generate a quadrivalent and thus be the cause of a chromosomal rearrangement following recombination.

Detection of Mosaicism

In 6/383 (1.6%) of patients we observe reduced dynamic range (signal intensity ratios with reduced deviation from log2 ratio of zero) for duplicated and deleted regions. These are listed in Table S1 (online supplementary information) along with details of the FISH (uncultured) and / or karyotype (cultured) confirmatory results. We also observe complete discordance between different foetal samples analysed. For foetus KUL 28, referred due to heart abnormalities, we received both an amniocentesis and foetal blood sample. A large amplification of the 16q arm was observed by array analysis on foetal blood but not in AF (both from DNA of uncultured cells). Karyotyping subsequently revealed an additional marker chromosome 16 in foetal blood but not in cultured amniocytes. Hence, this is a true foetal mosaicism for a marker chromosome 16q. Interestingly this finding would not have been identified if AF alone was received. In foetus G 2, a male foetus referred due to increased nuchal translucency, an intragenic 50kb deletion of the *DMD* gene was observed with reduced dynamic range, confirmed as mosaic by MLPA analysis on the same CVS DNA.

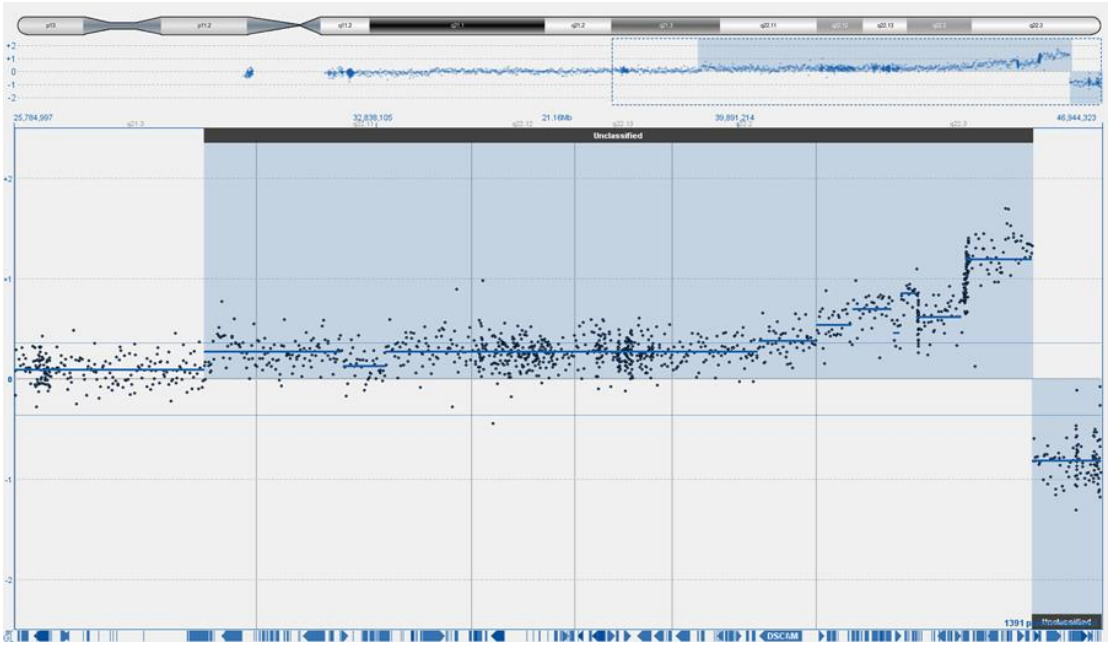

Figure 2. A complex rearrangement of chromosome 21. Duplication of 21q21.3q22.2, amplification of 21q22.2-q22.3, and terminal deletion of 21q22.3 are visible. Oligonucleotide probes are plotted by genomic position on the x-axis, and by normalised Log_2 signal intensity ratios on the y-axis. The duplicated and deleted regions are highlighted, with genes displayed in the lower track and chromosome 21 shown at top of image.

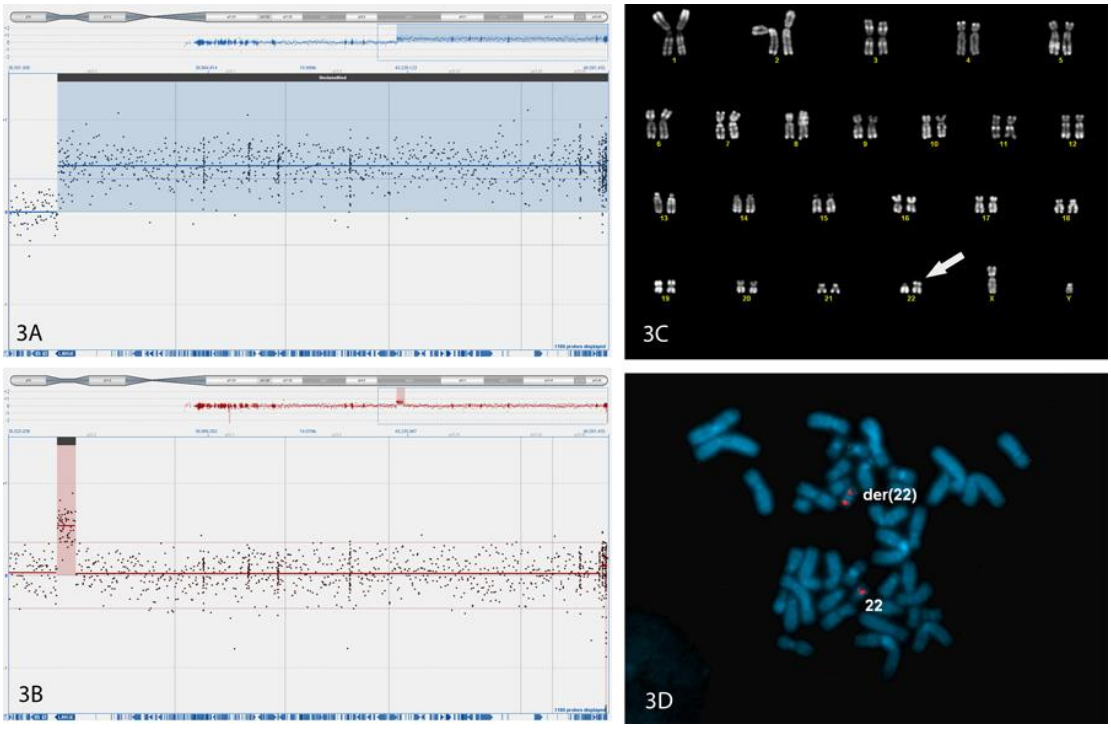

Figure 3. Paternal insertional translocation as driver of a chromosomal translocation. (a) A 17.5Mb duplication of 22q12.3-q13.33 in the foetus; (b) A 500kb duplication of 22q12.3 in the paternal sample. Oligonucleotide probes are plotted by genomic position on the x-axis, and by normalised Log_2 signal intensity ratios on the y-axis. The duplicated region is highlighted in (a) and (b), with genes

displayed in the lower track and chromosome 22 shown at top of image; (c) Displays the foetal karyotype with the derivative chromosome 22 (labelled with arrow); (d) Displays the FISH result in the paternal sample, using labelled BAC Clone RP11-413J08, showing 3 signals, with the additional signal on the derivative chromosome 22.

Subsequent analysis by array CGH and MLPA on both uncultured and cultured AF could not detect this microdeletion. Coincidentally, the conventional karyotype on both cultured CVS and AF revealed a balanced reciprocal translocation 46,XY,t(3;20)(q27;q11.2) inherited from the father. This translocation apparently has no direct phenotypic consequences, and no deletion of material was observed at the breakpoint by array analysis. Array CGH and MLPA performed postnatally on DNA from neonatal blood revealed no evidence of this microdeletion in the dystrophin gene. Hence, this represents a confined placental mosaicism for a *DMD* microdeletion.

Array Analysis of Apparently Balanced Rearrangements

In 2 familial cases of apparently balanced complex rearrangements, deletions are detected by array analysis at one or more of the breakpoints.

Variants of Uncertain Significance (VOUS)

Variants of uncertain significance were observed in 6/383 (1.6%) of foetuses. These are listed in Table S1 (online supplementary information), and consist of both inherited and *de novo* imbalances. In foetus KUL 37 the 2Mb deletion of 4q32 was shown to be inherited from the father. The same deletion was previously observed in an affected sibling. However, this child also had a *de novo* 17q deletion considered pathogenic. The deleted region on 4q32 harbours the *TLL1* gene, in which heterozygous mutations have been associated with atrial septal defects (Stanczak et al., 2009), and large deletions of 4q have also been reported in association with congenital heart defects (Xu et al., 2012). Since the father was already aware of this deletion, the variant was reported. For foetus G 7 the 2.6Mb deletion of 18q12.3, although relatively large and *de novo*, harbours only a single gene *PIK3C3*. Similar sized deletions have not been observed in normal individuals or patients. The *PIK3C3* gene is a candidate for schizophrenia, but its relation with the ultrasound findings in this foetus remains unclear. *PIK3C3* is known to regulate various intracellular membrane trafficking events. Zhou et al observed that *PIK3C3* is required for early embryogenesis and cell proliferation in mice (Zhou et al., 2011). Since this CNV was *de novo*, this was reported. In foetus KUL 34 we detected a 434 kb deletion of 15q11.2 in the PWAS BP1/BP2 region, subsequently also detected in the unaffected mother following parental array analysis.

Deletions of this region have been associated with developmental delay and behavioural problems as well as other features including schizophrenia (Doornbos et al., 2009; Sahoo et al., 2011). Strong phenotypic variability and reduced penetrance are characteristic of deletions of this region which make predictions of the future phenotype impossible. The most recent and largest study of Rosenfeld et al report deletions of this region having a penetrance risk of 10.4% (from 203/25,113 cases [0.81%], and 84/22,246 controls [0.38%]) (Rosenfeld et al., 2013). This CNV was not reported back to the patient based upon the lack of any family history of neurodevelopmental or psychological abnormalities, the low penetrance risk, and a lack of evidence for a link to the increased nuchal translucency observed and future penetrance of any phenotypic features. KUL33 was reported since this CNV occurred *de novo*, and also contains the *BRWD3* gene. Mutations in *BRWD3* (OMIM*300553) are associated with X-linked mental retardation (MR) and macrocephaly. Four copies of 2q21.3 were detected in KUL35, however no parental DNA was available. Only 6 genes are within the region, and hence this CNV was not reported. For KUL36, the referral reason of death in utero influenced the decision to report this VOUS with no known OMIM genes to the parents.

Discussion

Our findings demonstrate a 2.6% (1.0-4.2%, 95% confidence interval) increase in the diagnostic yield of causal genomic imbalances by discovery of pathogenic submicroscopic CNVs undetectable by conventional karyotype. Novel CNVs, as well as known microdeletion and microduplication syndromes were observed. Our results are slightly lower than those observed in a recent meta-analysis which found an increased diagnostic yield of chromosomal microarrays over karyotyping of 10% (8-13%, 95% confidence interval) in the presence of ultrasound anomalies (Hillman et al., 2013). Several recent large-scale prospective studies found rates between 6-8% in the presence of ultrasound anomalies (Shaffer et al., 2012; Breman et al., 2012; Wapner et al., 2012b). However, we demonstrate that arrays provide additional information over karyotyping in 3.9% of patients. Chromosomal microarrays: (i) provide more precise delineation of deletion and duplication breakpoints for structural rearrangements allowing for a more accurate assessment of gene content; (ii) accurately identify the origin of additional (euchromatic) chromosomal material; and (iii) can reveal pathogenic imbalances at the rearrangement breakpoints in apparently balanced rearrangements. Combining these findings gives a diagnostic yield of 6.5% above karyotyping alone, which is in line with other published studies. We also show that the

identification and localisation of CNVs can help in understanding the mechanism causing certain chromosomal rearrangements. This is important to estimate the recurrence risk for future pregnancies. A submicroscopic paternal CNV is an insertional translocation which confers a significant risk to future pregnancies and would have gone unnoticed by conventional karyotype alone. This highlights some previously reported and novel unexpected advantages of array analysis for the investigation of apparently balanced rearrangements.

We observed VOUS in 1.6% of patients, which included both inherited and *de novo* imbalances. Rates of VOUS ranged from 0.39%-4.2% dependent on whether *de novo* imbalances were included (Shaffer et al., 2012; Breman et al., 2012; Wapner et al., 2012b; Hillman et al., 2013). The risk of detecting a pathogenic CNV in the absence of any ultrasound anomalies has been estimated at between 0.5% and 1.7% in several studies which have included the use of chromosomal microarrays for general screening (Wapner et al., 2012b; Armengol et al., 2012; Lee et al., 2012). Hillman et al comment in their meta-analysis the high degree of heterogeneity in results of different array studies (Hillman et al., 2013), which may be due to a number of factors including the type of cohort studied and the type of platform used.

We demonstrate the ability to detect mosaicism of varying degrees and size by array analysis in 1.5% of patients, an important requirement if arrays are to be used in place of karyotype. Arrays provide an advantage over conventional karyotyping in the ability to measure mosaicism in DNA from uncultured material, providing an assessment of the level of mosaicism not influenced by the cell culture process. A recent study found mosaicism in a total of 1.2% of patients (n=43/3710) investigated by array and karyotype (Bi et al., 2012). Mosaicism was observed by karyotype alone in 39% of those mosaic cases (n=17/43); 11 of which showed very high levels (>80% mosaicism) and an abnormal array result, and 6 of which showed a normal array result due to very low levels (<10% mosaicism); 12% of those mosaic cases (n=5/43) were detected by array alone. Apparently balanced rearrangements were detected in 0.8% of cases (n=30/3710) (Bi et al., 2012). However, only approximately 6% of *de novo* apparently balanced rearrangements are considered to be pathogenic (Warburton, 1991), and studies have shown that approximately 40% of apparently balanced rearrangements harbour pathogenic CNVs at the breakpoints, or elsewhere (De Gregori et al., 2007; Schluth-Bolard et al., 2009). Taken together, it is clear that karyotyping and FISH remain essential tools for accurate prenatal genetic diagnosis, and parental cytogenetic

analysis may also be required to achieve a correct diagnosis of recurrence risk as demonstrated by our results and others. Karyotype is required for any family with a known family history of a balanced rearrangement only identifiable by conventional karyotype. In addition, those families with a prior abnormal pregnancy / child for which conventional karyotyping was not performed should also be eligible for karyotyping in order to exclude an unidentified balanced rearrangement as the cause.

Approach to genetic counselling:

Given that prenatal screening should be aimed at providing pregnant women with opportunities for meaningful reproductive choices, it is important to determine which types of information regarding CNVs may expand or otherwise even undermine these opportunities. Three main approaches to the reporting of information revealed by array analysis can be discerned. A first approach is to give patients the option during the pre-test counselling and informed consent process of which types of information they do / do not wish to be informed of following analysis (Siegal et al., 2012). A second approach is to reveal all information including the detection of VOUS. In a third approach, only information relating to the purpose of the analysis is provided (i.e. causal and/or clinically significant findings), and information deemed not of clear 'clinical relevance' based upon current knowledge is not reported to the patient (e.g. VOUS and inherited likely benign CNVs), who agrees to this during the pre-test counselling and informed consent process. This third approach is that adopted by the groups involved in this present study. All three approaches require thorough pre-test counselling (Wapner et al., 2012a; de Jong et al., 2011; Dondorp et al., 2012; McGillivray et al., 2012).

In recent years, case-control studies have enabled the quantification of the penetrance risk for certain recurrent microdeletions and microduplications, such as the 16p11.2 deletion and reciprocal duplication (Kaminsky et al., 2011; Cooper et al., 2011; Rosenfeld et al., 2013). Our approach is that those CNVs with penetrance risk factors below 25% were not reported back routinely, unless deemed to be clinically actionable. Examples of these situations include; the 15q11.2 deletion (*NIPA1* gene), with penetrance risk 10.4% which was detected in KUL34 and not reported; and the 22q11.2 duplication (*TBX1* gene), with penetrance risk 21.9% which was detected in KUL7 in association with cardiac anomalies; in the absence of cardiac anomalies on the clinical referral, this would nevertheless be reported and follow-up with detailed ultrasound examination for the presence of cardiac defects would be advised). The main difference between our approach and the laboratories providing all information

revealed by arrays to the pregnant women is that the latter will report back (1) rare/novel large (typically over 500 kb) inherited CNVs and (2) imbalances for known 'risk loci' where the future penetrance is uncertain and with low odds. Of note, in Mikhaelian et al's survey of prenatal genetic counsellors in the USA and Canada (Mikhaelian et al., 2013), the most prevalent ethical issue was the potential for ambiguous results, with 69% (n=111) of respondents expressing concerns, including about their ability to accurately interpret such results, the possibility a patient may terminate a pregnancy because of such results, and that such results may lead to psychosocial 'harm' to a patient.

Conclusion

Our results add support to other recent studies for the use of genomic arrays as the first-tier test for prenatal diagnosis of ultrasound abnormalities, as evidenced by the increased diagnostic yield of pathogenic submicroscopic imbalances as well as the additional information and faster reporting time offered in comparison to conventional karyotype. Considering the increased diagnostic yield in case of increased risk but in the absence of abnormal ultrasound, the use of chromosomal arrays as the first tier test for all invasive prenatal referrals seems warranted. Nevertheless, the workflow for classification of CNVs, the subsequent interpretation and reporting back of results warrants further societal research to determine the best provision of care.

Acknowledgments

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Patient	Phenotype	G.A. wks	Sample Type	Chr/cyto band	Size	Gain/Loss	No. of Genes [OMIM Genes]	Overlapping Syndrome	Inherited / de novo	Causal	Original karyotype result	Molecular karyotype Genome build hg18
Submicroscopic												
KUL1	Facial abnormalities	21	AF (C)	3q26.2-q26.31 15q11.2-q13.1	2.6 Mb 4.8 Mb	Deletion Duplication	21 [3] 100 [7]	PWAS	De novo De novo	Likely Yes	46,XY	arr 3q26.2-q26.31(172,166,063-174,754,887)x1 dn, 15q11.2-q13.1(21,248,261-26,067,061)x3 dn
KUL2	Ebstein malformation, cerebral ventriculomegaly	NR	AF (C)	1p36	5.3 Mb	Deletion	105 [4]	1p36 microdeletion	De novo	Yes	46,XX	arr 1p36.33-p36.31(622,816-5,901,907)x1 dn
KUL3	Mild ventriculomegaly	24	AF (UC)	7q22.1-q31.1	9.06 Mb	Deletion	200 [13]	Lissencephaly with cerebellar hypoplasia	De novo	Yes	46,XX,del(7)(q?)	arr 7q22.1q31.1(98,831,183-107,893,880)x1 dn
KUL4	Semi-lobar holoprosencephaly	14	CVS (UC)	10q24.31-q24.32	2.13 Mb	Deletion	56 [7]	SHFM-3	De novo	Yes	46,XY	arr 10q24.31q24.32(102,194,435-104,329,241)x1 dn
KUL5	Abnormal foetal central nervous system	16	AF (UC)	13q33.1-q34	10.51 Mb	Deletion	72 [8]		De novo	Yes	Not done	arr 13q33.1q34(103,614,991-114,123,757)x1 dn
KUL6	Familial ATRX dup	NR	CVS (UC)	Xq21.1	168 kb	Duplication	1 [1]		Maternal	Yes	Not Done	arr Xq21.1(76,703,047-76,870,877)x3 mat
KUL7	Complex cardiopathy	NR	AF (UC) & FB (UC)	22q11.2	2.5 Mb	Duplication	94 [7]	DGS/VCFS	De novo	Yes	46,XX	arr 22q11.21(17,270,162-19,771,944)x3 dn
KUL8	Polyhydramnios, cardiopathy, small stomach, short nasal bone	31	AF (UC)	22q11.2	2.6 Mb	Deletion	98 [7]	DGS/VCFS	De novo	Yes	Not done	arr 22q11.21(17.256.605-19.870.347)x1 dn
G1	Heart and renal anomalies	21	AF (UC)	22q11.21	2.5 Mb	Deletion	93 [7]	DGS/VCFS	De novo	Yes	46,XX	arr 22q11.21q11.21(17,274,835-19,794,060)x1 dn
G2	Increased NT (4,4 mm)	13	CVS (UC) & AF (UC)	Xp21.2p21.2	50 kb	Deletion	1 [1]	DMD	De novo	No CPM	46,XY,t(3;20)(q27;q27.2)pat	arr Xp21.2p21.2(31,134,695-31,184,180)x0 dn
Array CGH provides additional information over karyotype												
KUL9	MCA	NR	AF (C)	5pter-p13.3 9pter-p13.2	31.8 Mb 38.5 Mb	Deletion Duplication	146 [11] 494 [24]	CDC	De novo De novo	Yes Yes	46,XY,der(5)(p13;?)	arr 5pter-p13.3(69,497-31,811,114)x1 dn, 9pter-p13.2(198,982-38,526,895)x3

												dn
KUL10	MCA	NR	AF (C)	4p16.3-p16.2 4p16.2-p12	5.1 Mb 43.5 Mb	Deletion Duplication	97 [10] 266 [17]	WHS	De novo De novo	Yes Yes	46,XX,der(4)add(4)(p16)	arr 4p16.3-p16.2(34,021-5,135,978)x1 dn, 4p16.2-p12(5,187,465-48,761,351)x3 dn
KUL11	Cystic hygroma, IUGR	NR	AF (C)	4pter-p16.3 7pter-p21.3	1.8 Mb 16 Mb	Deletion Duplication	47 [5] 160 [5]	WHS	De novo De novo	Yes Yes	46,XX,add(4)(p16)	arr 4pter-p16.3(34,021-1,871,953)x1 dn, 7pter-p21.3(136,180-16,012,990)x3 dn
KUL12	Foetal hydrothorax	NR	AF (C)	17q21.31-q25.3 17q25.3	138 kb 36.3 Mb	Deletion Duplication	3 [0] 625 [55]	Campomelic dysplasia	De novo De novo	Yes Yes	46,XX,der(17)add(q25.3)	arr 17q21.31-q25.3(42,141,819-78,445,623)x3 dn, 17q25.3(78,513,526-78,651,671)x1 dn
KUL13	Cardiac defect, advanced maternal age	NR	AF(UC)	7q33-q36.3	22.3 Mb	Deletion	382 [20]	HPE3; Currarino	De novo	Yes	46,XX,del(7)(q33)	arr 7q33q36.3(136,496,304-158,817,239)x1 dn
KUL14	Increased NT (>2.5mm)	NR	AF (UC)	8q21.11-q22.3	24.8 Mb	Duplication	205 [11]		De novo		46,XY,der(8)add(8)(p?)	arr 8q21.11q22.3(77,820,698-102,626,232)x3 dn
KUL15	Cardiac defect	25	AF (UC)	22q12.3-q13.33	17.5 Mb	Duplication	364 [28]	Waardenburg syndrome 4; Phelan Mcdermid syndrome	Paternal Insertional translocation	Yes	46,XY,der(22)(qter->q12.3::p11.2->qter)	arr 22q12.3q13.33(32,069,468-49,567,789)x3 dn
G3	oligohydramnios, hypoplastic cerebellum, short femur	NR	AF (UC)	5p15.33p13.3 9q33.3q34.3	30.5 Mb 11.3 Mb	Deletion Duplication	141 [11] 280 [25]	CDC Walker Warburg syndrome; Tuberous sclerosis 1; Kleefstra syndrome	De novo	Yes	46,XX, der(5)t(5;9)(5p13.3;9q33.3)	arr 5p15.33p13.3(0-30,814,401)x1 dn, arr 9q33.3q34.3(129,771,347-141,053,475)x3 dn
G4	Flat facies & nasal bridge, holoprosencephaly	16	AF (UC)	18p11.32p11.21	14 Mb	Deletion	131 [3]	HPE4	De novo	Yes	45,XX,der(15)t(15;18)(q10;q10)	arr 18p11.32p11.21(108,760-14,685,777)x1 dn
G5	Congenital diaphragmatic hernia	30	AF (UC)	14q24.2q24.3	17 Mb	Deletion	157 [14]		De novo	Yes	46,XY,del(14)(q24.2q32.11)	arr 14q24.2q32.11(72,785,898-90,195,516)x1 dn
G6	Spontaneous pregnancy after ovarian failure	16	AF (UC)	8p23..2-p23.1 9q34.3	12 Mb 1.8 Mb	Duplication Deletion	167 [5] 90 [6]	8p23.1 deletion syndrome Kleefstra	De novo	Yes	46,XX,der(9)t(9;?)(q34;?)	arr 8p23.2p23.1(0-11,990,253)x3 dn, arr 9q34.3q34.3(138,338,249-140,138,746)x1 dn

								syndrome				
Complex rearrangements where array CGH reveals additional imbalances over karyotype												
KUL16	Known familial rearrangement	NR	AF (C)	6p12.3-p12.1 6q14.1	4.1 Mb 840 kb	Deletion Deletion	70 [6] 3 [0]		De novo De novo	Likely Unlikely	46,XX,t(4;13;6)(p15;q12;p12)	arr 6p12.3-p12.1(49,588,389-53,694,390)x1 dn, 6q14.1(77,421,309-78,266,274)x1 dn
KUL17	Abnormal FISH & Karyotype chr 21	13	CVS (UC & C)	21	q arm	Complex Mosaic	314 [20]		De novo	Yes	46,XX,der(21) .nuc ish(LSI21) x3	arr 21q21.3q22.2(29,719,730-40,252,214)x3 [40%], arr 21q22.2q22.3(40,287,127-45,586,329)x4, arr 21q22.3(45,608,516-46,920,235)x1
KUL18	Echogenic bowel, oligohydramnios	NR	AF (C)	2p21-p16.3 6q23.1-q23.2 11q24.3-qter 16q21 20p12.2-p12.1	3.49 Mb 663 kb 5.88 Mb 690 kb 1.71 Mb	Deletion Deletion Deletion Deletion Deletion	34 [6] 3 [0] 35 [2] 1 [0] 15 [2]	HNPCC Alagille syndrome	De novo De novo De novo De novo De novo	Likely Unlikely Likely Unlikely Likely	46,XX,t(2;16;6;20)(p21;q21;q23;p12)	arr 2p21p16.3(47,409,205-50,897,685)x1, arr 6q23.1q23.2(131,022,300-131,685,322)x1, arr 11q24.3q25(129,404,216-134,444,557)x1, arr 16q21(59,389,418-60,079,595)x1, arr 20p12.2p12.1(10,275,200-11,984,125)x1
KUL19	Hydrops, NT 6mm	NR	CVS (UC)	4p15.31-pter 8p23.3 8p23.2-23.1 8q24.11-qter 15q24.1-qter	18.75 Mb 2.13 Mb 8.38 Mb 27.36 Mb 27.70 Mb	Mosaic Dup Deletion Mosaic Del Mosaic Dup Mosaic Dup	204 [19] 12 [2] 128 [2] 252 [16] 340 [18]	WHS 8p23.1 deletion syndrome	De novo	Yes Yes Yes Yes Yes	46,XY,der(8)(qter->q24.11::p23->qter)	arr 4p16.3-p15.31(27,336-19,197,100)x3 [30%], arr 8p23.3-p23.2(176,785-2,311,066)x1, arr 8p23.2-p23.1(2,347,991-10,726,753)x1 [30%], arr 8q24.11-q24.3(118,902,689-146,102,353)x3 [30%], arr 15q11.2(19,108,674-19,433,141)x3 [30%]
Concordant with karyotype												
KUL20	Increased NT (4.2mm), absent nasal bone	14	CVS (UC)	22	Entire chromosome	Duplication	951 [56]		De novo	Yes	47,XY,+22	arr (22)x3 dn
KUL21	Hyperechogenic focus, left & right ventricles	NR	AF (UC)	13	Entire chromosome	Duplication (Mosaic)	884 [45]		De novo	Yes	47,XY,+13[4]/46,XY[5]	arr (13)x3 [60%] dn
KUL22	Cleft Lip & Cleft Palate,	21	AF (UC)	16p Xq12-qter	p arm; 35 Mb 89 Mb	Duplication Duplication	672 [49] 1222 [90]		De novo	Yes	Not Done	arr 16p13.3-p11.1(12,768-35,006,660)x3 dn, arr Xq12-q28(65,965,956-

												154,899,837)x2 dn
KUL23	ventriculomegaly, aortic dilation ascites	28	AF (UC)	9	Entire chromosome	Duplication (Mosaic)	1853 [92]		De novo	Yes	nuc ish(CEP9x3)[39]/(CEP9x2)[11] No karyotype	arr (9)x3[25%] dn
KUL24	Fryns Syndrome. Bilateral Hernia, VSD, MCA	18	AF (UC)	1q Yq	103 Mb 35.3 Mb	Duplication Deletion	1895 [116] 161 [2]		De novo	Yes	46,X,der(Y)t(Yq;1q)[11]/46,XY[4]	arr 1q21.1-qter(144,106,092-247,199,719)x3 [60%] dn, arr Yq11.223-qter(22,402,032-57,772,954)x0 [60%] dn
KUL25	Ventriculomegaly, ambiguous genitalia, midline defect	32	AF (UC)	X	Entire chromosome	Deletion (Mosaic)	2051 [159]		De novo	Yes	45,X[9]/47,XXX[1] .nuc ish(CEPXYx1)[19]/(CEPXYx3[68]/(CEPXYx2)[13]	arr (X)x1 [60%]/ arr (X)x2 [40%]
KUL26	Increased NT (4.4mm), hygroma colli, hydronephrosis	14	CVS (UC)	8q13.3-q24.13	55 Mb	Duplication	424 [24]		De novo	Yes	Not done	arr 8q13.3-q24.13(70,747,122-126,110,305)x3 dn
KUL27	MCA, heart defect	11	CVS (UC)	16p13.3-q24.3	Entire chromosome	Duplication	1202 [98]		De novo	Yes	Not done	arr 16p13.3q24.3(24,543-88,794,888)x3 dn
KUL28	AVSD, Unbalanced left hypoplastic heart	23	Foetal blood (UC) & AF (UC)	16q22.1-q24.3	23.59 Mb	Amplification	360 [33]		Both parents have fragile chr 16 (mosaic)	Yes	46,XX (on AF sample) 47,XX,i(16)(q10) (on fetal blood)	arr 16q22.1q24.3(65,101,656-88,690,913)x4
KUL29	MCA	20	AF (UC)	13	Entire chromosome	Duplication	884 [45]		De novo	Yes	47,XX,+13	arr 13q12.11q34(18,400,387-114,123,757)x3 dn
KUL30	Death in utero	16	CVS (UC)	21q11.2-q22.3	32.5 Mb	Duplication	419 [23]		De novo	Yes	47,XX,+21	arr 21q11.2q22.3(14,400,995-46,920,235)x3 dn
KUL31	Microcephaly, microphthalmia	30	AF (UC)	13	Entire chromosome	Duplication	884 [45]		De novo	Yes	47,XY,+13	arr 13q11q34(18.348.559-114.123.757)x3 dn
Incidental findings unrelated to phenotype												
G2	Paternal balanced translocation inherited by foetus	13	CVS (UC) & AF (UC)	3; 20					NOT TESTED		46,XY,t(3;20)(q27;q27.2)pat	
KUL32	Paternal iso(Xq) NOT PRESENT IN FOETUS			Xq					NOT TESTED		46,XY	arr Xq11qter(61,848,425-154,883,082)x3 [40%]
Unclassified Variants												
KUL33	Arthrogryposis,	19	AF (UC)	Xq21.1	352 kb	Duplication	6 [1]		De novo	VOUS	46,XX	arr Xq21.1(79,664,552-

	hypokinesia, Cerebral malformation											80,017,026)x3 dn
KUL34	Increased NT (7mm)	13	CVS (UC)	15q11.2	434 kb	Deletion	6 [1]	15q11.2 PWAS BP1-BP2 microdeletion	Maternal	VOUS	Not Done	arr 15q11.2(20,305,022-20,739,252)x1 mat
KUL35	Cerebral abnormalities, cerebellar hypoplasia	18	AF (UC)	2q21.3	656 kb	Amplification	6 [1]		Father not available	VOUS	Not Done	arr 2q21.3(135,436,770-136,093,172)x4
KUL36	Death in utero	21	AF (UC)	15q26.1	747 kb	Duplication	5 [0]		Paternal	VOUS	Not Done	arr 15q26.1(91,253,495-92,000,368)x3 pat
KUL37	Microdeletion in sister	12	CVS (UC)	4q32.3	2.165 Mb	Deletion	21 [1]		Paternal	VOUS	Not Done	arr 4q32.3(165,525,955-167,691,444)x1 pat
G7	Cerebral and ocular abnormalities, median cleft, duodenal atresia	25	AF (UC)	18q12.3q12.3	2.6 Mb	Deletion	3 [0]		De novo	VOUS	46,XY	arr 18q12.3q12.3(35,697,785-38,169,137)x1 dn

Supplementary Table S1.

AF=amniotic fluid sample; CVS=chorionic villus sample; (C)=Cultured cells; (UC)=uncultured cells; NT=nuchal translucency; CPM=confined placental mosaicism; MCA=multiple congenital anomalies; IUGR=intrauterine growth retardation; VSD=ventricular septal defect; AVSD=atrioventricular septal defect

PWAS=Prader-Willi / Angelman syndrome; SHFM-3=Split Hand / Foot Malformation-3; DGS/VCFS=DiGeorge syndrome / Velo-cardio-facial syndrome; DMD=Duchenne Muscular Dystrophy; CDC=Cri Du Chat syndrome; WHS=Wolf Hirschhorn syndrome; HPE3=Holoprosencephaly 3; HPE4=Holprosencephaly 4; DSCR=Downs syndrome critical region; HPE1=Holoprosencephaly 1; HNPCC=Hereditary non-polyposis colon cancer;

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Chapter 4

Application of Chromosomal Microarrays for Gene Discovery in Congenital Diaphragmatic Hernia

Chapter 4. Application of Chromosomal Microarrays for Gene Discovery in Congenital Diaphragmatic Hernia

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Targeted array comparative genomic hybridisation (array CGH) identifies genomic imbalances associated with isolated congenital diaphragmatic hernia (CDH).

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Abstract

Objective: Congenital diaphragmatic hernia (CDH) is a congenital birth defect affecting around 1/3000 births. We propose that a significant number of isolated CDH cases have an underlying genetic cause, and that a subset of these result from copy number variations (CNVs) identifiable by array CGH. *Methodology:* We have designed a custom array targeted at genes and genomic loci associated with CDH. 79 isolated CDH patients were screened using this targeted array. *Results:* In 3 patients we detect genomic imbalances associated with the observed diaphragmatic hernia; a deletion of 8p22-p23.3, 14.2Mb in size, a 340kb duplication of Xq13.1 including the ephrin-B1 gene (*EFNB1*), and mosaicism for trisomy 2. *Conclusion:* Using this approach, we detect genomic imbalances associated with CDH in 3/79 (4%) isolated CDH patients. Our findings further implicate 8p deletions as being associated with CDH. The duplication of *EFNB1* further highlights this gene as a potential candidate involved in diaphragm development. Mosaicism for trisomy 2 is a rare event and unlikely to be a common cause of CDH. Further investigations of isolated CDH patients by array CGH will continue to identify novel submicroscopic loci and refine genomic regions associated with CDH.

Introduction

Congenital Diaphragmatic Hernia (CDH) is a congenital birth defect with an incidence of around 1/3000 births (Torfs et al., 1992; Skari et al., 2000). CDH can be anatomically divided into three main hernia subtypes; a posterolateral 'Bochdalek' hernia in around 70% of cases, an anterior 'Morgagni' hernia in around 27% of cases, and a central septum transversum hernia in around 3% of cases. The vast majority of hernias are left sided (85%), whilst the remainder are right sided (13%) or bilateral (2%) (Torfs et al., 1992; van Loenhout et al., 2009; Pober, 2007). Next to the diaphragm defect, the lung developmental anomaly is an essential part of the phenotype. It results in variable degrees of pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality and morbidity in survivors.

CDH occurs as an isolated defect in around 50% of cases, or as non-isolated CDH for the remainder in which additional congenital malformations are present (Stoll et al., 2008). Non-isolated CDH is associated with abnormalities in a number of other systems including; cardiovascular system (27.5%), urogenital system (17.7%), musculoskeletal system (15.7%), and central nervous system (9.8%) (Stoll et al., 2008). It may also occur as part of a recognised syndrome for which a single causal gene may be identified. Examples include; *STRA6* in Matthew-Wood syndrome (Golzio et al., 2007; Pasutto et al., 2007; Chassaing et al., 2009; Segel et al., 2009), *WT1* in Denys-Drash syndrome (Devriendt et al., 1995; Antonius et al., 2008), and *EFNB1* in craniofrontonasal syndrome (Wieland et al., 2004; Vasudevan et al., 2006). Alternatively, non-isolated CDH may be associated with specific genetic loci, including 8p23.1 (Faivre et al., 1998; Wat et al., 2009), and 15q26 (Klaassens et al., 2005; Slavotinek et al., 2006; Klaassens et al., 2007), or with a clinically recognised syndrome of currently unknown genetic cause such as Fryns syndrome (Fryns et al., 1979; Fryns et al., 1989; Neville et al., 2002; Slavotinek, 2004).

It is still uncertain whether genetic factors are the sole cause of CDH or if they merely provide a genetic susceptibility to abnormal diaphragm development in concert with other environmental influences. The dual-hit hypothesis has been proposed, which speculates that the lung abnormality is a primary developmental defect, firstly affecting both lungs before diaphragm development and subsequently affecting the ipsilateral lung after defective diaphragm development (Keijzer et al., 2000). Chromosomal abnormalities have been reported to be present in as much as 30% of individuals with CDH (Howe et al., 1996; Tonks et al., 2004; Thorpe-Beeston et al., 1989; Geary et al., 1998; Pober, 2007) and it has been

postulated that these cytogenetic abnormalities can provide positional information about the genomic locations for CDH-causing genes.

In view of the relative rarity of Mendelian pedigrees in families with CDH, isolated CDH is considered to have multi-factorial inheritance with gene mutations, environmental factors, gene-gene and gene-environment interactions contributing to exceed a threshold in susceptible individuals. A number of candidate genes are involved in the retinoic acid pathway (Montedonico et al., 2008; Klaassens et al., 2009). Previous research using animal models has demonstrated that environmental factors including vitamin A deficient (VAD) diet or exposure to teratogenic agents, particularly nitrofen, can disrupt the retinoid signalling pathway resulting in CDH (Wilson et al., 1953; Thebaud et al., 1999; Babiuk et al., 2004; Oshiro et al., 2005; Nakazawa et al., 2007b; Nakazawa et al., 2007a; Noble et al., 2007; Clugston et al., 2010a). Further evidence of the involvement of this pathway comes from knockout or mutant mouse models in which genes involved in retinoid signaling are shown to cause CDH when silenced, including compound null retinoic acid receptor (RAR) mutants which were shown to have a spectrum of VAD-like defects including diaphragm defects (Lohnes et al., 1994; Mendelsohn et al., 1994; Lohnes et al., 1995) .

For the majority of CDH patients, particularly isolated cases, the genetic cause remains unknown. It has been shown recently that copy number variations (CNVs) underlie many genetic disorders and that a subset of all monogenic diseases is caused by CNVs rather than mutations. The causal genes for isolated CDH in humans remain largely unknown, and given the large number of potential candidate genes and pathways, mutation screening may be of limited value at this time. One approach to identify the genes involved in diaphragm development is to study genomic regions recurrently associated with CDH patients. The technique of array comparative genomic hybridisation (array CGH) is one such method which has demonstrated the ability to refine the critical regions associated with various disorders, including CDH (Klaassens et al., 2005; Slavotinek et al., 2006; Shaffer et al., 2007; Scott et al., 2007).

As opposed to applying genome-wide cytogenomic arrays which have an average resolution of 30-50kb (Miller et al., 2010), a targeted array would enable higher resolution coverage of regions of interest, and as a consequence be able to uncover smaller CNVs in relevant genes. In order to identify novel genetic causes of isolated CDH, we have therefore developed a targeted oligonucleotide array. This array is designed to provide coverage of genomic regions recurrently associated with non-isolated CDH in humans, supplemented by candidate

genes associated with diaphragm and lung development. We hypothesised that by applying high resolution targeted array comparative genomic hybridisation (array CGH) to cases of isolated CDH we would identify causal genomic imbalances, refine genomic regions causal for CDH, and identify candidate genes important for diaphragm development.

Methods

Patients

A total of 88 fetuses with isolated CDH, who were managed in the prenatal period by the fetal medicine units from the University Hospital Gasthuisberg, Leuven, Belgium (n=72), and the Hospital Clinic-IDIBAPS, University of Barcelona and Centre for Biomedical Research on Rare Diseases (CIBER-ER), Barcelona, Spain (n=16), were analysed using the targeted array. Samples from Leuven were either stored genomic DNA extracted from cultured amniotic fluid (AF) (n=49), or frozen AF cultures from which genomic DNA was extracted following thawing and re-culturing (n=16), or genomic DNA extracted from neonatal cord blood (n=7). Those from Barcelona (n=16) were uncultured AF samples which had been frozen, and from which genomic DNA was later extracted. Only those patients in whom the absence of additional major congenital anomalies was confirmed at birth were considered eligible for inclusion in this study. All samples were collected with approval of the respective local ethical committee, and in accordance with the Helsinki Declaration of 1975 (as revised in 1983).

Targeted Array Design

An array was designed comprising 15,000 (15K) oligonucleotide probes in an 8x 15K format (Oxford Gene Technology [OGT], Oxford, UK). The approach for the array design is a combination of two methods; 1. To target the genomic regions recurrently associated with CDH in humans. 2. To target candidate genes from CDH animal models and proposed pathways involved in CDH. (See results section, and supplementary tables S1 & S2).

Array Comparative Genomic Hybridisation (array CGH)

Reference genomic DNA samples were derived from healthy males and females. All patients and sex-matched reference samples were labelled with fluorescent Cy-3 and Cy-5 dyes in dye-swap experiments. The CytoSure Genomic DNA Labelling Kit (OGT) was used according to the manufacturer's protocol, with the following modifications; 500ng DNA was labelled

instead of 1000ng, and consequently all reagent volumes were halved. The DNA denaturation step was performed at 98°C for 20 minutes, and the DNA digestion step was omitted. The subsequent incubation at 37°C was performed overnight. For DNA purification spin columns provided with the CytoSure Genomic DNA Labelling Kit (OGT) were used according to the manufacturer's protocol. For concentration of labelled DNA samples, either Clean & Concentrator-5 spin-columns (Zymo Research, CA, USA) or Amicon Ultra-0.5 spin columns (Millipore, MA, USA) were used according to the manufacturer's protocol. Hybridisation mixes were prepared using the Oligo aCGH Hyb kit (Agilent Technologies S.A./N.V., Belgium) according to the manufacturer's protocol. Hybridisation was performed using SureHyb chambers and a rotating oven (Agilent Technologies) at 65°C for 24 hours as recommended. Following hybridisation, arrays were washed manually according to the manufacturer's protocol using Agilent Wash Buffer 1 and Wash Buffer 2, following by rinsing and drying using acetonitrile.

Arrays were scanned immediately with a DNA Microarray Scanner (Agilent Technologies) at 3 micron resolution. The tif images were visually checked for artefacts. Signal intensities were generated using the Feature Extraction software (v.10.5.1. Agilent Technologies). To visualise the data and perform analysis the CytoSure Interpret Software (OGT) was used. A global Lowess normalisation is applied by the software in order to correct for the dye incorporation bias.

Data Analysis

The CytoSure Interpret software package features a Circular Binary Segmentation (CBS) algorithm, where thresholds were set at +/- 0.36 from a Log_2 ratio of zero, and at least 5 flanking probes within a segment. For a region to be called as aberrant it must be present in both datasets within the dye-swap experiment. To complement the CBS algorithm, a second threshold-based method was also applied to the individual probe Log_2 ratios. Data from the patient labelled in Cy5 and in Cy3 is combined as follows; $X^2 \times Y^2 = Z$ (where, X = individual probe normalised Log_2 ratio for Cy5-labelled patient data, Y = individual probe normalised Log_2 ratio for Cy3-labelled patient data). The calling threshold was then set at 5 flanking probes where $Z > 0.016$.

Therefore, by applying a threshold of 5 probes, we achieve an average resolution for detecting imbalances of ~80 kb for target regions, and ~16 kb for target genes.

The CytoSure Interpret software package (OGT) was used for interpretation of the CNVs detected. We have previously characterised the CNVs present in our male and female control samples at ~200kb resolution provided by the CytoSure Syndrome Plus v2 105K array (OGT), as well as created an internal database of common likely benign CNVs. Data from the Toronto Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/) was also used to aid the interpretation of CNVs detected. Any CNV detected in a patient and also within regions in our internal benign CNV database were removed from further investigation. Any CNV detected in a patient which were within regions containing at least two reports in the DGV of being copy number variable in normal individuals were also excluded from further investigation.

Confirmation of imbalances was achieved by the use of a genome-wide 105K array (CytoSure Syndrome Plus v2, OGT), or by FISH. All genomic coordinates are based upon the NCBI 36 genome assembly build.

Results

A custom array was designed to target genomic loci and genes associated with CDH. 19 genomic regions were selected covering 225Mb for which there were at least three cases of CDH in humans identified from a literature search (including (Holder et al., 2007)). For the target genomic regions the average probe spacing is 1 probe every 20kb. 75 CDH-associated genes covering 17Mb were selected following a literature search, complemented by using aGeneApart (www.esat.kuleuven.be/ageneapart). aGeneApart uses a text-based input to data-mine the literature and identify genes which display an association. For our purposes the search term “congenital hernia of diaphragm” (LNDB 170403) was used. Candidate genes were selected based upon; single gene disorders associated with CDH in humans, genes identified from animal models of CDH, genes whose expression was dysregulated in CDH models, and genes involved in cellular pathways proposed to be involved in CDH. Probe coverage included 5kb flanking region for target genes. Average probe coverage for target genes is 55 probes per gene (range 17-95 probes), with average probe spacing of 1 probe every 4kb. Target genomic regions and genes selected for this array are listed in supplementary tables S1 and S2, respectively.

A targeted array comprising 15,000 oligonucleotide probes was designed to provide high resolution coverage of genomic regions recurrently associated with non-isolated CDH, as well as candidate genes for diaphragm and lung development. Eighty-eight isolated CDH patients

were screened for genomic imbalances through the use of the custom-design targeted array. Nine samples (10.23 %) had insufficient quantity of DNA for analysis, or poor quality DNA for which the result could not be interpreted. For the remaining 79 patients, 76 patients (96.2%) displayed normal results, i.e. no CNVs, or only benign CNVs were detected. For three patients (3.8%) genomic imbalances were detected; a deletion of chromosome 8p22-p23.3 in one patient, a duplication of Xq13.1 encompassing the *EFNB1* gene in a second patient, and mosaicism for trisomy 2 in a third patient.

Foetus 1

The mother was referred because of the presence of a left-sided CDH with herniation of the liver into the thorax. The Observed to Expected Lung to Head Ratio (O/E LHR) was 21.8%. No other abnormalities were detected except for a missing 12th rib. Fetoscopic tracheal occlusion (FETO) was performed. The gestational age at the time of diagnosis was 27 weeks. The child was delivered at 36 weeks gestation, with birth weight 2300 grams, and deceased soon after with all clinical features of pulmonary hypoplasia but no other abnormal features. Autopsy was not accepted by the parents, however, a postnatal echocardiography was performed for which no cardiac defects were observed. The array result showed an interstitial deletion on chromosome 8p22-p23.3. This deletion was confirmed by a genome wide 105K array (CytoSure Syndrome Plus v2, OGT), which also refined the proximal deletion breakpoint. The deletion was shown to be 14.2Mb in size (958312 – 15160490bp) and is shown in figure 1.

Foetus 2

The mother was referred at 27 weeks because of the presence of a left-sided CDH and liver herniation. The O/E LHR was 22%. No other abnormalities were detected. FETO was undertaken. The male child was delivered at 38 weeks gestation, birth weight 2585 grams, with CDH and no other observed abnormalities. The child died due to pulmonary hypoplasia and pulmonary hypertension. The array result showed a duplication of Xq13.1, including the EFNB1 gene. This duplication was confirmed by a genome wide 105K array (Cytosure Syndrome Plus v2, OGT), which also refined the duplication breakpoints. The duplication is 340 kb in size (67899816 – 68240036bp) and is displayed in figure 2. Parental (maternal) samples were declined.

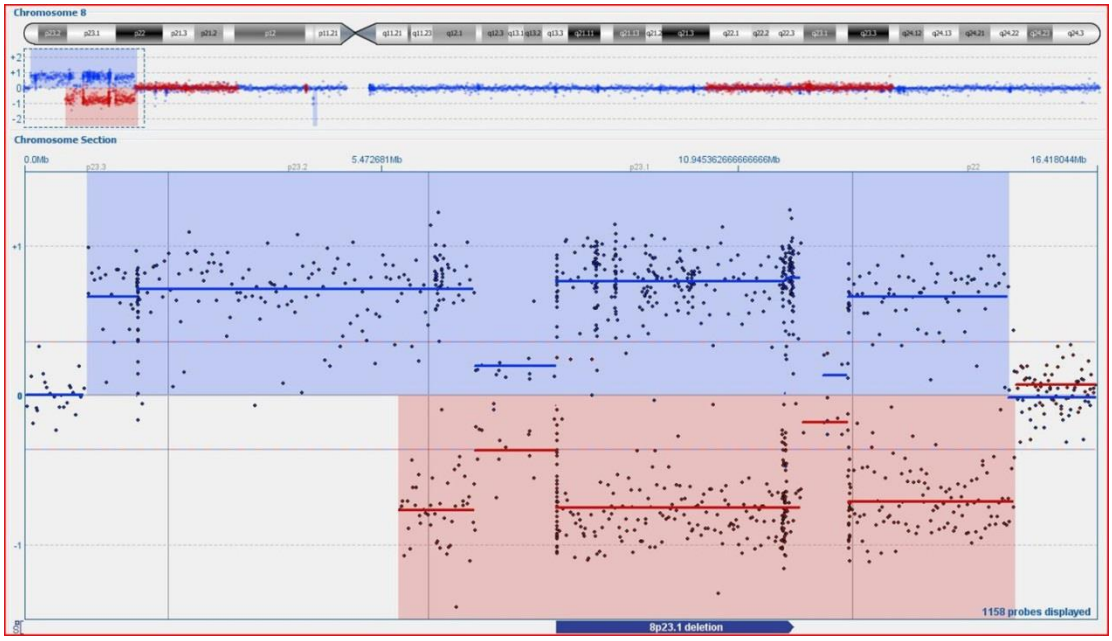

Figure 1. Chromosome 8p23.3-p22 deletion; Red dataset displays the 15K custom array result for patient labelled in Cy5, and blue dataset displays the 105K genome-wide array result for patient labelled in Cy3. The 105K array result determined the telomeric break point of the deletion. The location of the 8p23.1 deletion region is shown at the bottom of the image.

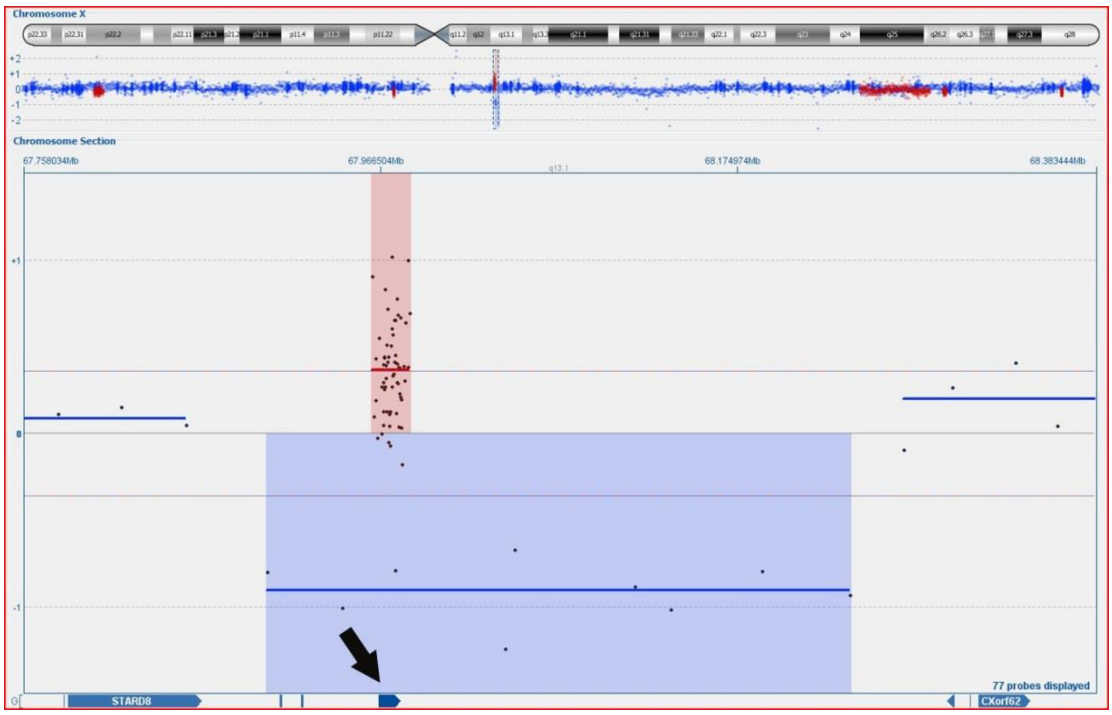

Figure 2. EFNB1 gene duplication on X chromosome; Red dataset displays the 15K custom array result for patient labelled in Cy5, and blue dataset displays the 105K genome-wide array result for patient labelled in Cy3. The 105K array refines the extent of the duplication. Genes are displayed in the blue track, where the black arrow highlights the position of the EFNB1 gene.

Foetus 3

The mother was referred because of the presence of a left-sided CDH, without liver herniation, and moderate lung hypoplasia (O/E LHR = 29.8%). No other abnormalities were detected. Fetoscopic tracheal occlusion (FETO) was performed within the TOTAL trial. The male child died postnatally from pulmonary hypoplasia, with no additional congenital malformations observed. Autopsy was declined. The array result indicated a high level of mosaicism for trisomy 2. This result was confirmed by a genome wide 105K array (Cytosure Syndrome Plus v2, OGT). The average median signal intensity ratios for probes on chromosome 2 for the targeted array and for the genome-wide array were found to be 0.35 and 0.39 respectively, suggesting 60 - 97% mosaicism. FISH analysis with a commercially available centromeric FISH probe (CEP2 SpectrumOrange VYSIS, Abbott S.A./N.V., Belgium) on cultured AF cells determined the level of mosaicism to be ~88%, with 391/445 trisomic cells observed. The array result and the FISH confirmation are displayed in figure 3A & 3B.

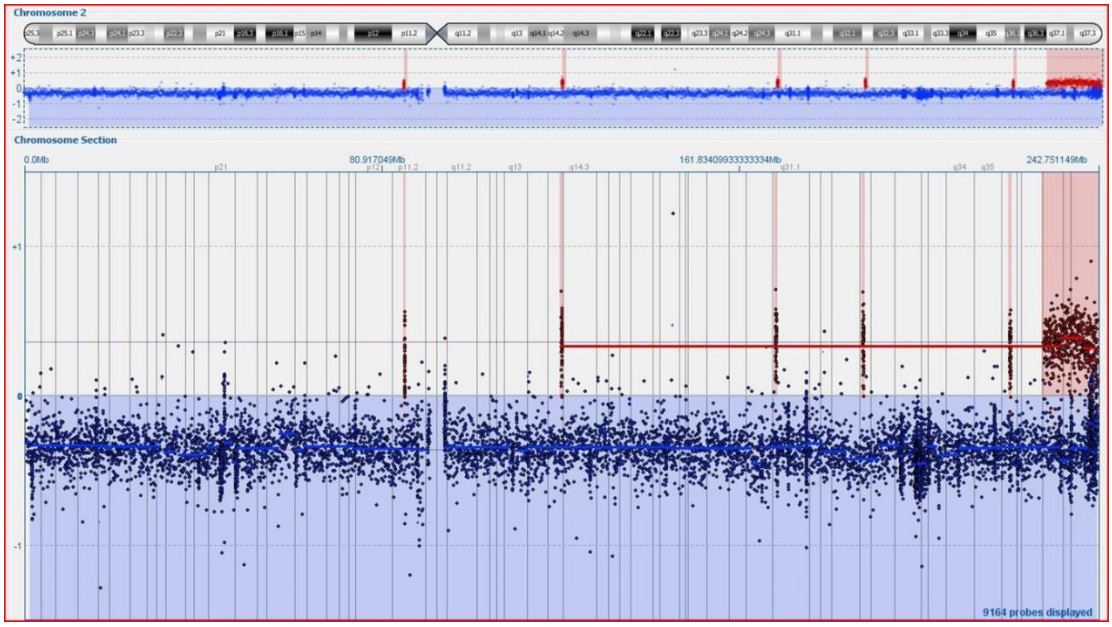

Figure 3A. Mosaic trisomy 2; Red dataset displays the 15K custom array result for patient labelled in Cy5, and blue dataset displays the 105K genome-wide array result for patient labelled in Cy3. The entire chromosome 2 is shown, with the duplication apparent.

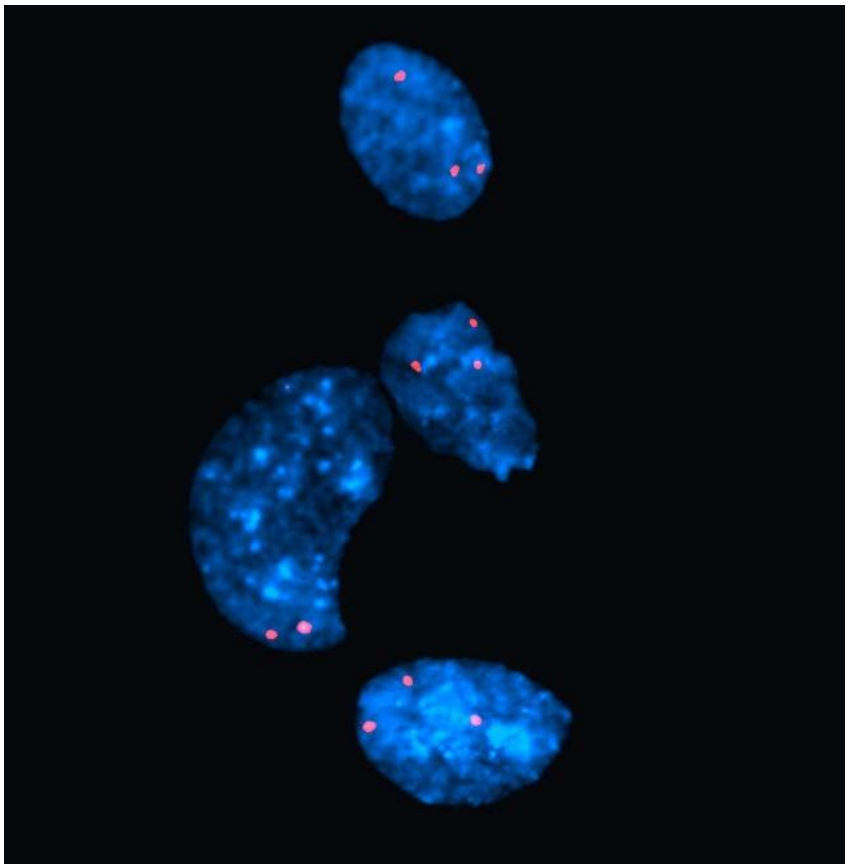

Figure 3B. Mosaic Trisomy 2; Displays an image from the confirmatory FISH analysis used to determine the level of mosaicism. A centromeric FISH probe (CEP2 SpectrumOrange VYSIS, Abbott) was used, where three or two fluorescent red signals equal a cell with three or two copies of chromosome 2 respectively.

Discussion

We designed a targeted array comprising 15,000 oligonucleotide probes. This array was targeted at regions recurrently associated with CDH in humans, and to potential candidate genes involved in diaphragm and lung development. A screen of 79 isolated CDH patients using this targeted oligonucleotide microarray revealed 2 causal imbalances and 1 likely causal variant.

The finding of an 8p deletion in one patient adds further evidence to this region being associated with CDH. 8p deletions encompassing the 8p23.1 microdeletion region have previously been observed in a number of patients with CDH (Pecile et al., 1990; Howe et al., 1996; Faivre et al., 1998; Borys and Taxy, 2004; Slavotinek et al., 2005; Shimokawa et al., 2005; Lopez et al., 2006; Baynam et al., 2008; Wat et al., 2009). In addition, deletions of this region are also associated with congenital heart defects (Claeys et al., 1997; Devriendt et al., 1998; Devriendt et al., 1999). *GATA4* is considered to be the gene responsible for the heart abnormalities observed in patients with 8p deletions following the identification of *GATA4* mutations in patients with congenital heart defects (Garg et al., 2003; Okubo et al., 2004; Tomita-Mitchell et al., 2007; Rajagopal et al., 2007; Reamon-Buettner and Borlak, 2005; Reamon-Buettner et al., 2007). *GATA4* has also been previously proposed as a candidate gene for diaphragm defects given the diaphragmatic abnormalities observed in mice heterozygous for a deletion of *GATA4* exon 2 (Jay et al., 2007). However, no patients have been reported with *GATA4* mutations and CDH. Interestingly, our patient was an apparently isolated case of CDH, with no heart defects detected prenatally by ultrasound, or postnatally by echocardiography. To our knowledge there are no reported cases of 8p deletions in association with isolated CDH. However, we cannot fully exclude the presence of a heart defect in our patient, and additional phenotypic features of typical 8p23.1 microdeletion syndrome may not be apparent at birth.

The transcription factor *COUP-TFII* has been shown to interact with *FOG2* (Huggins et al., 2001), which in turn modulates the transcriptional activity of *GATA4* (Crispino et al., 2001). *COUP-TFII* and *FOG2* are located on 15q26 and 8q23 respectively, regions recurrently associated with CDH in humans reviewed in (Holder et al., 2007)). Furthermore, tissue-specific ablation of *COUP-TFII* in mice caused a posterolateral Bochdalek-type CDH (You et al., 2005), and *FOG2* N-ethyl-N-nitrosourea (ENU) mutant mice displayed diaphragm and lung defects (Ackerman et al., 2005). All of these genes are proposed to be involved in the retinoic acid pathway, and their co-expression in the mesenchymal cells of the

pleuroperitoneal folds supports the retinoid hypothesis and the mesenchymal hit hypothesis which have been proposed as potential mechanisms of abnormal diaphragm development (Clugston et al., 2006; Jay et al., 2007; Clugston et al., 2010a). It is possible that these proteins act together to regulate transcription of target genes downstream of the retinoic acid pathway involved in normal diaphragm development. There may be additional genes related to diaphragm development present in the deleted region. However, with no evidence supporting other candidate genes in the 8p region as being involved in CDH, more research is needed. The exact mechanism by which 8p deletions cause diaphragm defects is unknown, and the influence of *GATA4* deletions or mutations on human CDH remains to be proven.

Mutations and deletions of the EFNB1 gene have been linked to craniofrontonasal syndrome (CFNS) (Twigg et al., 2004; Wieland et al., 2005; Wieland et al., 2007), in which diaphragmatic defects have been observed in a number of patients (Vasudevan et al., 2006). CFNS is an X-linked disorder in which the phenotype is of greater severity in heterozygous females than in hemizygous males. The mechanism by which mutations in *EFNB1* manifest themselves is proposed to occur by cellular interference (Wieacker and Wieland, 2005; Twigg et al., 2006; Wieland et al., 2008). In females, due to random X-inactivation there are 2 sub-populations of cells, some of which express the wild type and some the mutant EFNB1 allele, supported by in vitro evidence from studies of CFNS patient cell lines (Wieland et al., 2008). This process is proposed to cause a cellular interference which for EFNB1 interferes with tissue boundary formation (Twigg et al., 2004). Recently, Twigg et al identified somatic mosaic mutations in more severely affected CFNS males compared to constitutionally affected males (Twigg et al., 2013) further supporting the increased severity in heterozygous females with a cellular interference model. Since our male patient displays a previously unreported duplication of Xq13.1 including the *EFNB1* gene, we consider this imbalance to be an unclassified variant. Unfortunately, maternal samples were not available to determine the inheritance status of this imbalance. Whilst the syndromic features of CFNS are seen with greater severity in affected females, since CDH is observed in both males and females with *EFNB1* mutations, it is unlikely that the cellular interference model can explain abnormal diaphragm development without the presence of somatic mosaic males. The exact function of *EFNB1* with respect to diaphragm development is unknown. EFNB1 acts as both a receptor and a ligand in a tissue-specific manner during embryogenesis, and the resulting forward and reverse signalling may be important for diaphragm development. *EFNB1* may display a degree of dosage sensitivity in which duplications, as well as deletions

or mutations, affect the function of developing tissues. *EFNB1* has important roles in migration of neural crest cells, a pathway proposed to be involved in CDH (Davy et al., 2004; Davy and Soriano, 2005; Arvanitis and Davy, 2008). A single report in the database of genomic variants (DGV) finds the region encompassing the *EFNB1* gene to be a rare variant in normal individuals (frequency <1%) (Shaikh et al., 2009). However, this finding has not been reproduced by other studies of normal individuals and, hence, may be an artefact. Furthermore, we have not observed a similar CNV in over 5000 cases meanwhile analysed by array CGH in our laboratory. Duplications of *EFNB1* may represent a susceptibility locus for CDH, or may be a rare but benign CNV. Without the identification of additional isolated CDH patients with similar duplications, or further imbalances from studies of normal individuals, it is difficult to fully establish the clinical significance of this finding.

Mosaicism for trisomy 2 is a rare finding with only 7 liveborn cases reported thus far (Robinson et al., 1997; Sago et al., 1997; Mihci et al., 2009). In a recent report, Mihci *et al* describe a patient with mosaic trisomy 2 and displaying mental retardation, multiple congenital anomalies including diaphragmatic hernia, and dysmorphic features similar to Pallister-Killian syndrome (Mihci et al., 2009). Our patient therefore represents the eighth reported case of mosaic trisomy 2 in a liveborn, and the second case with CDH. The finding of CDH in our patient adds further evidence to this abnormality being part of the variable spectrum of phenotypic features associated with mosaicism for trisomy 2. Imbalances on chromosome 2 have previously been observed in CDH patients, reviewed by Holder, et al. (Holder et al., 2007), although our finding does not make it possible to further confirm these specific regions as being causal for CDH. However, given the rarity of this finding, we conclude that whilst CDH does display an association with mosaic trisomy 2, this finding does not represent a common cause of diaphragmatic hernia.

We have taken the approach of only targeting the regions recurrently associated with non-isolated CDH, as well as targeting CDH-associated genes. All patients had a karyotype performed, as well as FISH analysis for the common aneuploidies (21, 18, 13, X & Y), which revealed normal results. FISH analysis to exclude the presence of isochrosome 12p which causes Pallister-Killian syndrome was also performed for the majority of patients revealing normal results. Only 19 patients have had a genome-wide array performed which revealed only benign CNVs for these patients. Therefore, we cannot rule out that submicroscopic imbalances outside of our target regions may be present in some patients. Additional research of both isolated and non-isolated CDH patients by array CGH is likely to reveal

novel submicroscopic loci associated with CDH, and refine those regions recurrently associated with CDH.

Conclusion

We identified chromosome abnormalities causal for CDH in one patient with an 8p deletion and in one patient mosaic for trisomy 2. In addition, the duplication of *EFNB1* further highlights this gene as a potential candidate involved in diaphragm development. Since a number of loci which display strong associations with CDH are below the resolution of conventional karyotyping, such as 8p23.1 deletions, 15q26 deletions, and 4p16 deletions (Wolf-Hirschhorn syndrome), the continued investigation of isolated CDH patients by high resolution microarrays will eventually reveal the true incidence of submicroscopic imbalances as a cause of CDH, and whether imbalances in genomic loci associated with syndromic CDH are also a cause of isolated CDH. Gene prioritisation strategies and next-generation sequencing may also prove to be an effective method of screening a large cohort of isolated CDH patients for mutations in the many candidate genes which are associated with CDH.

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Identification of dosage sensitive genes in foetuses referred with severe isolated congenital diaphragmatic hernia

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Abstract

Objective: Congenital Diaphragmatic Hernia (CDH) is a foetal abnormality affecting diaphragm and lung development with a high mortality rate despite advances in foetal and neonatal therapy. CDH may occur either as an isolated defect or in syndromic form for which the prognosis is worse. Although conventional karyotyping and more recently chromosomal microarrays support a substantial role for genetic factors, causal genes responsible for isolated CDH remain elusive. We propose that chromosomal microarray analysis will identify CNVs associated with isolated CDH.

Methods: We perform a prospective genome wide screen for copy number variations using chromosomal microarrays on 75 foetuses referred with apparently isolated CDH, six of which were later reclassified as non-isolated CDH.

Results: The results pinpoint haploinsufficiency of *NR2F2* as a cause of CDH and cardiovascular malformations. In addition, the 15q25.2 and 16p11.2 recurrent microdeletions are associated with isolated CDH. By using gene prioritisation and network analysis we provide strong evidence for several novel dosage sensitive candidate genes associated with CDH.

Conclusions: Chromosomal microarray analysis detects submicroscopic copy number variations associated with isolated CDH or CDH with cardiovascular malformations.

Introduction

Congenital Diaphragmatic Hernia (CDH) is a congenital abnormality affecting diaphragm and lung development with an incidence of 1.7 to 5.7 per 10,000 live-born infants (Kotecha et al., 2012). CDH occurs either as an isolated defect or in syndromic form for which the prognosis

is worse (Skari et al., 2000). CDH is associated with variable degrees of pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality rate, and much of the morbidity observed in survivors. Survival rate of isolated CDH is in the range of 60-70% and better in high-volume centres (Kotecha et al., 2012).

Animal models have been used to identify many genes involved in diaphragm development (Clugston et al., 2006; van Loenhout et al., 2009; Brady et al., 2011). Some of those genetic models are tissue-specific or incorporate hypomorphic alleles which allow investigators to study diaphragm development in cases for which complete elimination of gene function using traditional knockout mice results in early lethality (Ackerman et al., 2005; You et al., 2005; Jay et al., 2007; Wat et al., 2012). Mouse and rat models have also revealed the importance of the pleuroperitoneal folds (PPFs) in diaphragm development and how defects in this structure can lead to CDH (Greer et al., 2000; Babiuk et al., 2003; Clugston et al., 2010b; Mayer et al., 2011). Patterns of gene expression have been studied using transcriptome analysis in both normal and teratogenic animal models identifying dysregulated genes and pathways (Clugston et al., 2010a; Russell et al., 2012; Dingemann et al., 2012). Among the most important pathways that have been identified are the retinoic acid (RA), SHH, WNT and TGFβ signalling pathways as genetic variants within these pathways are shown to cause CDH in humans and / or animal models (Kim et al., 2001; Clugston et al., 2010a; Russell et al., 2012).

Analogous with those animal models, CDH is a key feature of different human monogenic syndromes. Gene mutations causing syndromic forms of CDH in humans include; *STRA6* [OMIM *610745] (Matthew-Wood syndrome); *NIPBL* [OMIM *608667] (Cornelia de Lange syndrome), *WT1* [OMIM *607102] (Denys-Drash syndrome); and *LRP2* [OMIM *600073] (Donnai-Barrow syndrome). In addition to single gene mutations, large chromosomal imbalances are also a frequent cause of syndromic CDH. Conventional karyotyping and, more recently, genome-wide screening for copy number variations (CNVs) has identified many loci scattered throughout the genome that are recurrently deleted or duplicated in individuals with CDH. Those regions most likely harbour one or more genes for which diaphragm development is dosage sensitive. Chromosomal regions commonly identified in association with CDH include; 8p23.1 [OMIM %222400], 8q23 [OMIM #610187], 15q26 [OMIM %142340], and 1q41-42 [OMIM #612530] which is a possible locus for Fryns syndrome (Klaassens et al., 2005; Kantarci et al., 2006; Shaffer et al., 2007; Klaassens et al., 2007; Scott et al., 2007; Wat et al., 2009; Kantarci et al., 2010; Srisupundit et al., 2010;

Rosenfeld et al., 2011; Wat et al., 2011). Those results show that the use of genome wide arrays enable the identification of dosage sensitive genes involved in human CDH. In addition to syndromic forms, CDH is often seen as an isolated defect, and those patients are most suited to prenatal and/or neonatal therapy to improve lung growth and function (Jani et al., 2009; Deprest and De Coppi, 2012). Despite a large body of evidence that genetic variants can cause diaphragmatic hernias in animal models, confirmation of their involvement in human *isolated* CDH is largely lacking.

We hypothesised that by applying chromosomal microarrays to a prospective study of isolated CDH foetuses we would identify pathogenic copy number variations (CNVs) which would allow for; (i) identification of novel CNVs and genes linked to CDH; and (ii) identification of causal genes by refinement of known CDH loci. As a European reference centre for foetal therapy during the prenatal phase following identification of isolated severe CDH, we have acquired detailed phenotype information and have access to a large cohort of patients.

Methods

Patients

Pregnant women with foetuses affected by severe isolated CDH were referred to UZ Leuven for prenatal assessment and where applicable evaluation for possible Fetoscopic Endoluminal Tracheal Occlusion (FETO) during pregnancy. The presence of any major anomaly for which the pathology of this alone would indicate a poor prognosis is classified as non-isolated; 'isolated' CDH may include minor anomalies which are not a contraindication for foetal management or a prognostic factor for outcome or response to therapy. Severity of herniation was determined by measurement of the normalised lung area to head circumference ratio (LHR) and sonographic evidence of intra-thoracic herniation of the liver as previously described (Jani et al., 2009). All patients received pre-test counselling and provided informed consent for chromosomal microarray analysis. Samples were received between July 2009 and December 2012. Approval for this study was granted by the KU Leuven / University Hospital Leuven Commission for Medical Ethics (S55513).

Chromosomal Microarray Analysis

Genome-wide array comparative genomic hybridisation (array CGH) analysis was undertaken for all cases on DNA extracted from amniotic fluid (AF), either directly or from

cultured cells. For 1 case an additional foetal blood sample was also analysed (foetus 7, see Results section). Samples were analysed using a 180K chromosomal microarray (Oxford Gene Technology, UK) in dye swap experiments with patient DNA labelled with Cy5 hybridised against a sex-matched commercial reference DNA sample (Megapool Reference DNA, Kreatech Diagnostics, The Netherlands) labelled with Cy3, and vice versa. Analysis and visualisation of array results was achieved using the CytoSure Interpret software (Oxford Gene Technology, UK), with analysis based upon genome build hg18. CNVs were firstly filtered against an in-house curated database of common polymorphic CNVs. Classification is subsequently aided by tracks for external databases such as the DGV (Database of Genomic Variants, http://projects.tcag.ca/variation/), ISCA database (The International Standards for Cytogenomic Arrays Consortium, www.iscaconsortium.org) and DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources, http://decipher.sanger.ac.uk/). DNA extracted from parental blood samples was also analysed using the same platform to assist with interpretation of pathogenic and/or rare CNVs detected in the foetus.

Confirmation of findings

Rare CNVs were confirmed by dye-swap analysis using the same chromosomal microarray platform, fluorescent *in situ* hybridisation (FISH), or conventional karyotyping, as appropriate.

Prioritisation of Genes Located Within CNVs & Network Analysis

Gene prioritisation was performed to prioritise and rank candidate genes within CNV regions detected by array analysis. A training set of genes was curated for CNV gene prioritisation which was performed using Endeavour (www.esat.kuleuven.be/endeavour). Full details of the training gene set used and the CNV prioritisation results are provided in online supplementary information. Evidence for involvement of candidate genes in CDH was further aided by determining; (i) whether candidate genes are reported as dysregulated in animal models and/or human CDH [gene list updated from (Brady et al., 2011)]; (ii) whether candidate genes are expressed in the normal developing PPFs and / or developing muscular diaphragm [gene lists sourced from (Russell et al., 2012)]; and (iii) whether candidate genes interact directly with genes in a CDH gene network. This CDH gene network was created using Ingenuity Pathway Analysis software package (IPA), (Ingenuity Systems, CA, USA). The 'training genes' were connected where experimental evidence of direct interactions existed

in the software database; i.e. predicted interactions were not permitted. To this 'CDH gene network', we connected candidate genes from the CNV loci identified in this study, again only where direct interactions were supported by experimental evidence in the software database. In more general terms, the 'training genes' which are associated with CDH were used as 'baits' to 'fish' for interacting candidate genes within our CNV loci. Genes located within the marker ring X and 13q were not incorporated since these contain too many genes and would diffuse the identification of causative genes.

A network analysis of this CDH gene network was then performed within the IPA software in order to identify which biological functions and pathways are enriched for. The network scores are derived from p-values (score = -log p-value) and represent the likelihood that focus genes within the network are enriched for by random chance alone. Similarly, the p-values associated with a function or a pathway is a measure of the likelihood that the association between a set of focus genes in and a given process or pathway is due to random chance alone. The p-values are calculated using the right-tailed Fisher Exact Test. Full details are provided in online supplementary information. Further details of the methodology and the results are provided in the Supporting information.

Results

75 consecutive foetuses referred due to severe, and apparently isolated CDH were analysed prospectively. Of those 75 foetuses, a total of 69 foetuses had *isolated* CDH; 67 of which were confirmed clinically as isolated CDH (15 of those by necropsy or MRI virtopsy); for 2 foetuses no necropsy, MRI virtopsy or postnatal clinical information was available. Six foetuses considered isolated on initial prenatal examination were later shown to be *non-isolated* CDH at subsequent examinations; 1 from necropsy or MRI virtopsy, and 5 from clinical observation (1 of which was identified postnatally).

Genomic array analysis revealed *de novo* CNVs in 9.3% (7/75 foetuses) and rare inherited variants which may be involved in CDH in a further 4% (3/75 foetuses). The full clinical and molecular cytogenetic findings are provided in Table 1. None of those patients presented in Table 1 underwent necropsy or MRI virtopsy. Clinical confirmation of isolated CDH was confirmed postnatally in 4 (foetuses 2, 3, 8 and 9). Two pregnancies were terminated, with no necropsy or MRI performed (foetus 5 and foetus 10). Foetus 1 had a left clubfoot and hydrops; foetus 4 had hydrops. These foetuses were all considered to have *isolated* CDH.

For 2 foetuses (foetus 6 & foetus 7), a coarctation of the aorta was identified and were re-classified as *non-isolated* CDH.

Foetus 7 was referred due to isolated CDH, however, a coarctation of the aorta was later identified. A deletion of 15q26.2, only 1.7Mb in size (94,221,024-95,920,254; build hg18), was detected on DNA from uncultured AF. The mean Log2 signal intensity ratio was -0.6, which indicates the presence of a mosaicism where the deletion would be present in ~60% of cells. Chromosomal microarray on DNA from a subsequent (uncultured) foetal blood sample revealed an identical result. Parental array analysis showed no CNV at this locus, confirming this deletion originated *de novo*. Conventional karyotype on cultured AF revealed 46,XX in 2/10 cells and 47,XX,+mar in 8/10 cells. However, the array analysis did not reveal any large regions of additional genomic material, suggesting that the marker chromosome consists of heterochromatic material only. This finding thus represents the smallest reported deletion of the 15q26 locus in a patient with CDH and cardiovascular malformation (CVM) (Figure 1). The figure also shows the former CDH 'smallest region of overlap' at 15q26, ~5 Mb in size, identified by FISH and microarray analysis, and the positions of the specific deletions which defined this region (Klaassens et al., 2005; Castiglia et al., 2005; Slavotinek et al., 2006; Klaassens et al., 2007).

In foetus 10 with left-sided CDH and a single umbilical artery, array CGH on DNA from uncultured AF showed a deletion of the X chromosome, however, a 10Mb region of Xp22.2p22.11, and a 15Mb region of Xp11.1q13.1 showed reduced dynamic range of signal intensity ratios. In order to confirm this unusual array finding, FISH analysis was performed with probes for the X centromere and the Xp sub-telomere regions (CEPX & TelXpYp) in a dual hybridisation, and for probes targeted to the Xp22 and Xq11 regions (RP11-1129A6 & RP11-284B18) in a dual hybridisation. This revealed the presence of 1 signal in 23/50 and 2 signals in 27/50 cells for the centromeric X chromosome probe, but only a single signal for the Xp sub-telomeric probe in the same 50 cells. Probes for the Xp22 and Xq11 regions both displayed a single signal for 40/50 cells counted and 2 signals for 10/50 cells. The karyotype on cultured AF revealed the presence of a 45,X cell line in 20/25 cells and a 46,X,+ring chromosome of unidentified origin, in 5/25 cells examined. The array, karyotype and FISH results are displayed in Figure 2. The final interpretation based upon array and conventional cytogenetic investigations was of a ring X chromosome comprised of material from Xp22.2p22.11 and the Xp11.1q13.1 centromeric region, present in mosaic form, and lacking the X inactivation centre (XIC).

	Chr/cyto band	size	Gain/Loss	CDH Type	Additional Features	Inherited / de novo	Classification	Karyotype result	Array Result Genome build hg18	FETO	Outcome
Foetus 1	1p22.2	102 kb	Duplication	Bilateral	Left clubfoot, hydrops	Paternal	VOUS	46,XY	arr 1p22.2(92,027,785-92,129,888)x3, pat	Y	Deceased
Foetus 2	4p15.2-p14	13.487 Mb	Duplication	Right	None	De novo	Pathogenic	46,XY	arr 4p15.2p14(22,819,570-36,306,519)x3, dn	N	Alive
Foetus 3	13q12.12	1.37 Mb	Duplication	Left	None	Paternal	VOUS	46,XX	arr 13q12.12(22,439,339-23,808,774)x3, pat	N	Alive
Foetus 4	13q14.3-q33.3	57 Mb	Mosaic Duplication	Left	Hydrops	De novo	Pathogenic	Not performed	arr 13q14.3q33.3(51,620,432-108,613,709)x3 [30%], dn	Y	Deceased
Foetus 5	15q25.2	1.592 Mb	Deletion	Left	None	De novo	Pathogenic	Not performed	arr 15q25.2(81,011,052-82,602,711)x1, dn	N	TOP
Foetus 6	15q25.2 17p12	2.434 Mb 1.344 Mb	Deletion Deletion	Left	Coarctation of the aorta	De novo De novo	Pathogenic VOUS	46,XX	arr 15q25.2(80,422,638-82,856,949)x1, dn 17p12(14,039,023-15,382,903)x1, dn	Y	Deceased
Foetus 7	15q26.2	1.699 Mb	Deletion	Left	Coarctation of the aorta	De novo	Pathogenic	46,XX[2]/47,XX,+MAR[8]	arr 15q26.2(94,221,024-95,920,254)x1 [60%], dn	N	Deceased
Foetus 8	16p11.2	543 kb	Deletion	Left	None	De novo	Pathogenic	Not performed	arr 16p11.2(29,559,861-30,102,955)x1, dn	Y	Alive
Foetus 9	17p13.3	713 kb	Duplication	Left	None	Maternal	VOUS	46,XY	arr 17p13.3(1,656-714,933)x3, mat	Y	Deceased
Foetus 10	Xp22.2-p22.11 Xp11.1-q13.1	10.4 Mb 15.4 Mb	Mosaic Ring X	Left	SUA	De novo	Pathogenic	46,X,r(X)(::p22.2p22.11::p11.1q13.1::) [5] / 45,X [20]	arr Xp22.33p22.2(106,314-12,656,628)x1, dn Xp22.2p22.11(12,741,959-23,093,838)x2 [30%], dn Xp22.11p11.21(23,293,215-56,523,984)x1, dn Xp11.1q13.1(56,573,141-71,997,108)x2 [30%], dn Xq13.2q28(72,063,396-154,899,837)x1	N	TOP

Table 1. Molecular cytogenetic results and clinical summary of CDH foetuses with abnormal chromosomal microarray findings. VOUS=Variant of Uncertain Significance; FETO=fetoscopic endoluminal tracheal occlusion; TOP=termination of pregnancy; SUA=single umbilical artery

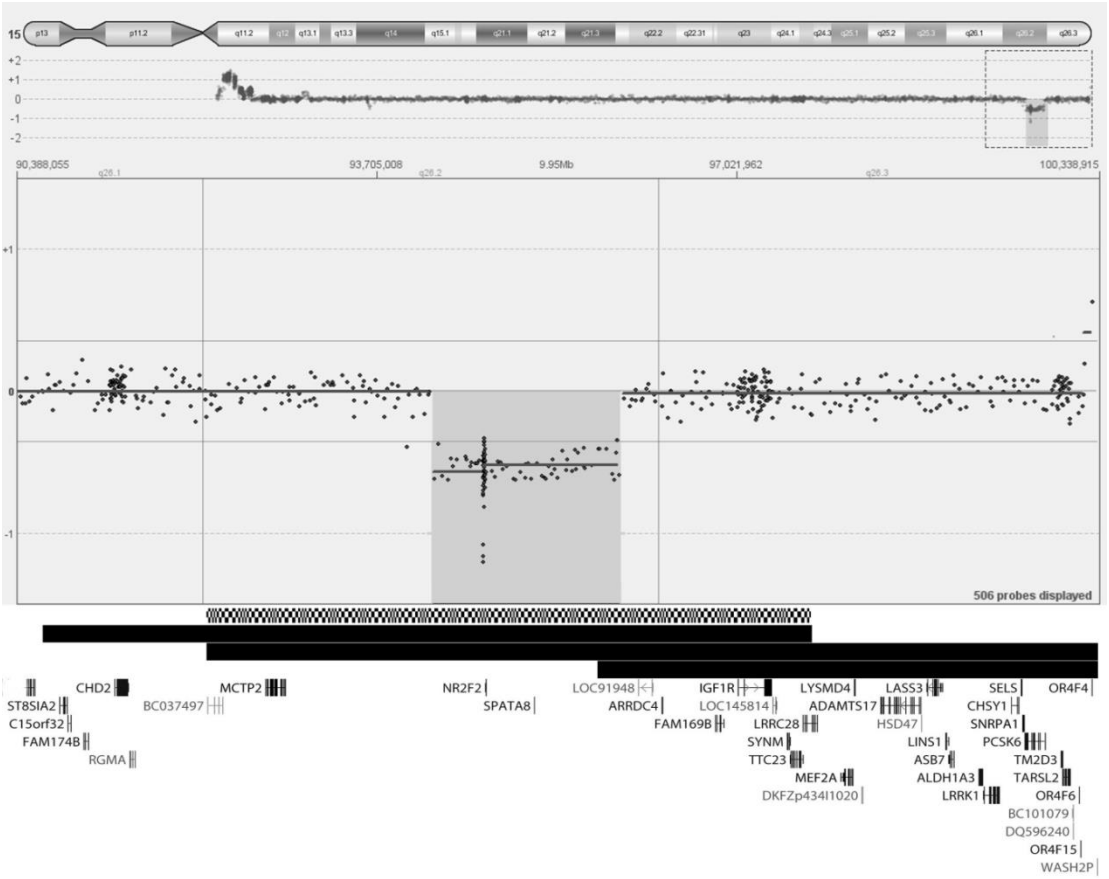

Figure 1. Refinement of the critical deleted region at 15q26 with a single case of isolated CDH. The array result of foetus 7 is displayed in the main window with probes plotted by genomic position on the x-axis and signal intensity log ratio on the y-axis. The tracks below display (from top to bottom); (i) the previous smallest region of overlap (checkered track); (ii) Patient C and (iii) Patient H from Klaassens et al 2007, which allowed for defining the region in shown in (i); and (iv) Patient 2 reported by Mosca et al 2011, which was recently used to refine the 15q26 region, however, this case is the result of an unbalanced translocation with a 50Mb duplication of 2p. (v) Protein coding genes from refseq visualised within the UCSC genome browser are shown below, where NR2F2 and SPATA8 are the only genes located within the deleted region in foetus 7.

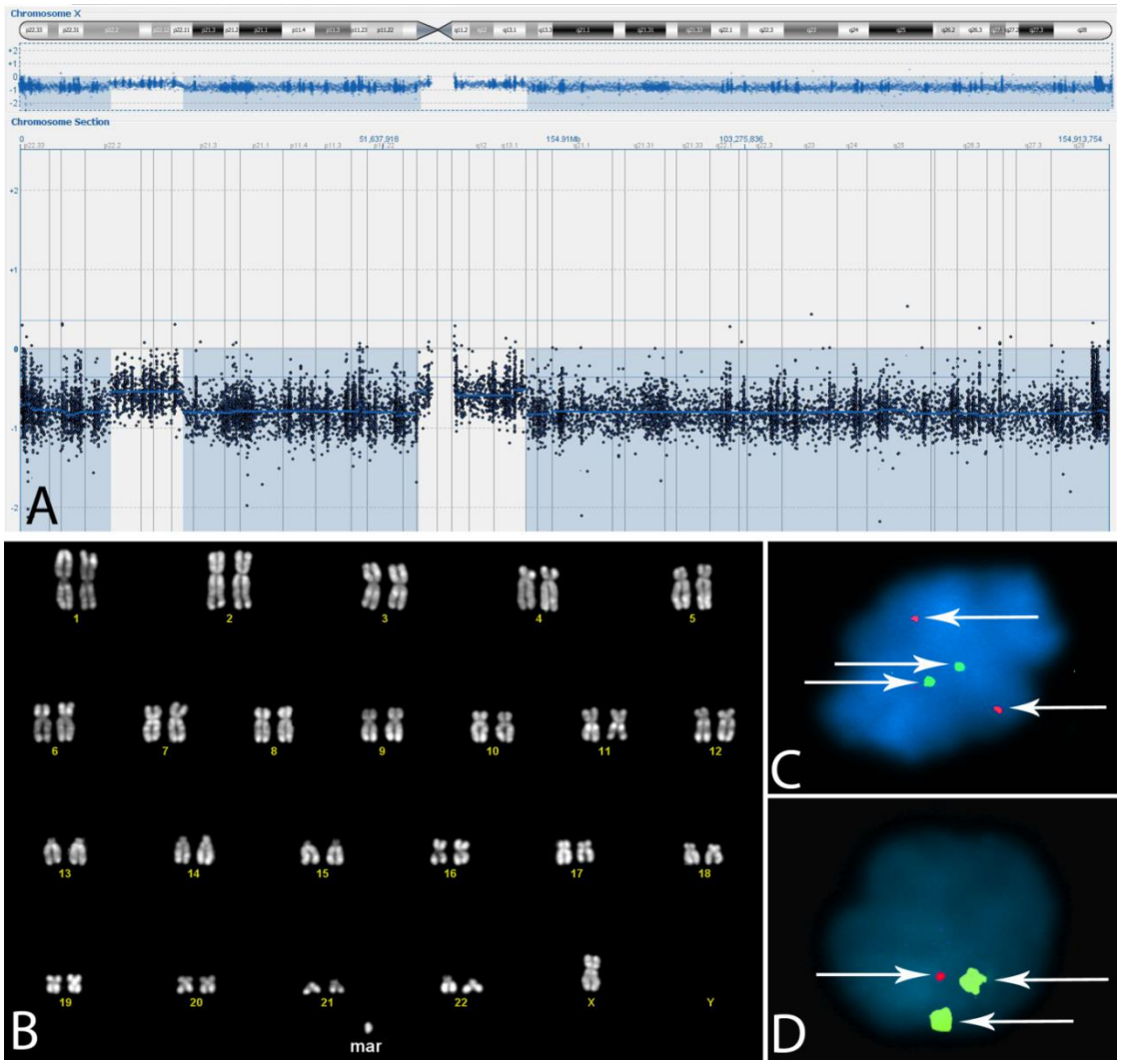

Figure 2. Cytogenetic analysis of foetus 10. A. The array result for chromosome X is shown in the main window with the deleted region highlighted. Probes are plotted by genomic position on the x-axis and signal intensity ratio on the y-axis. The reduced deviation of Log2 signal intensity ratios is apparent for the Xp22.2-p22.11 and Xp11.1-q13.1 regions. B. The karyotype is shown which revealed a 46,X, +mar cell line in a proportion of cells examined. C. FISH result with probes for the Xp22 and Xq11 regions (RP11-1129A6 & RP11-284B18, respectively) revealed 2 signals for both probes in a proportion of cells examined (left arrows = Xp22, right arrows = Xq11). D. FISH result with probes for the X centromere and the Xp sub-telomere regions (CEPX & TelXpYp) revealed the presence of 2 signals for the centromeric X chromosome probe, but only a single signal for the Xp sub-telomeric probe in a proportion of cells examined (left arrow = TelXpYp, right arrows = CEPX).

Foetuses 5 and 6 both have *de novo* deletions of 15q25.2, 1.59Mb and 2.43Mb in size, respectively. These deletions overlap the 15q25.2 microdeletion syndrome locus in which CDH has been observed, as well as cognitive deficits and Diamond--Blackfan anaemia (Wat et al., 2010). Foetus 5 had isolated left-sided CDH; foetus 6 was referred due to isolated left-sided CDH, though a coarctation of the aorta was detected later in pregnancy. Foetus 6 also has a *de novo* duplication of 17p12, 1.34Mb in size (Charcot-Marie-Tooth disease Type 1A locus).

A *de novo* deletion of 16p11.2, 543kb in size, was detected in foetus 8 with isolated left-sided CDH on DNA from uncultured AF. This coincides with the recurrent 16p11.2 microdeletion syndrome [OMIM #611913], in which 2 cases of CDH were recently reported (Wat et al., 2011). This locus is however more often associated with autism spectrum disorder and schizophrenia (Itsara et al., 2009; Sahoo et al., 2011).

A *de novo* duplication of 4p15.2-p14, 13.5Mb in size, was detected in foetus 2 with isolated right-sided CDH on DNA from uncultured AF. Given the size, *de novo* nature, gene content, and lack of reports in normal individuals this CNV is likely to be pathogenic.

Aside from *de novo* events, we also detect rare inherited variants whose pathogenicity is uncertain. Given the lack of reports of similar CNVs in normal individuals, these may represent risk loci for CDH. These include; a 102kb paternally inherited duplication of 1p22.2 which partially overlaps the *TGFBR3* gene, detected in foetus 1 with bilateral CDH; a paternally inherited duplication of 13q12.12, 1.37Mb in size, in foetus 3 with left-side CDH; and, a maternally inherited 713kb duplication of 17p13.3 in foetus 9 with left-side CDH.

Gene Prioritisation

We performed gene prioritisation using the Endeavour application (Tranchevent et al., 2008). Full details of these 'training genes' is provided in online supplementary information, along with the ranked gene prioritisation results of each CNV loci. Using the 'training genes', we curated a 'CDH gene network' using the Ingenuity Pathway Analysis software application. This curated 'CDH gene network' is displayed in Figure 3, and shows ; (i) the presence of training genes in CNVs identified in this study and previous studies of CDH, e.g. *NR2F2, GATA4, ZFPM2*, and *EFNB1*; and (ii) the presence of genes in CNVs identified in this study which interact directly with the CDH gene network, e,g *RBPJ*, and *BNC1*.

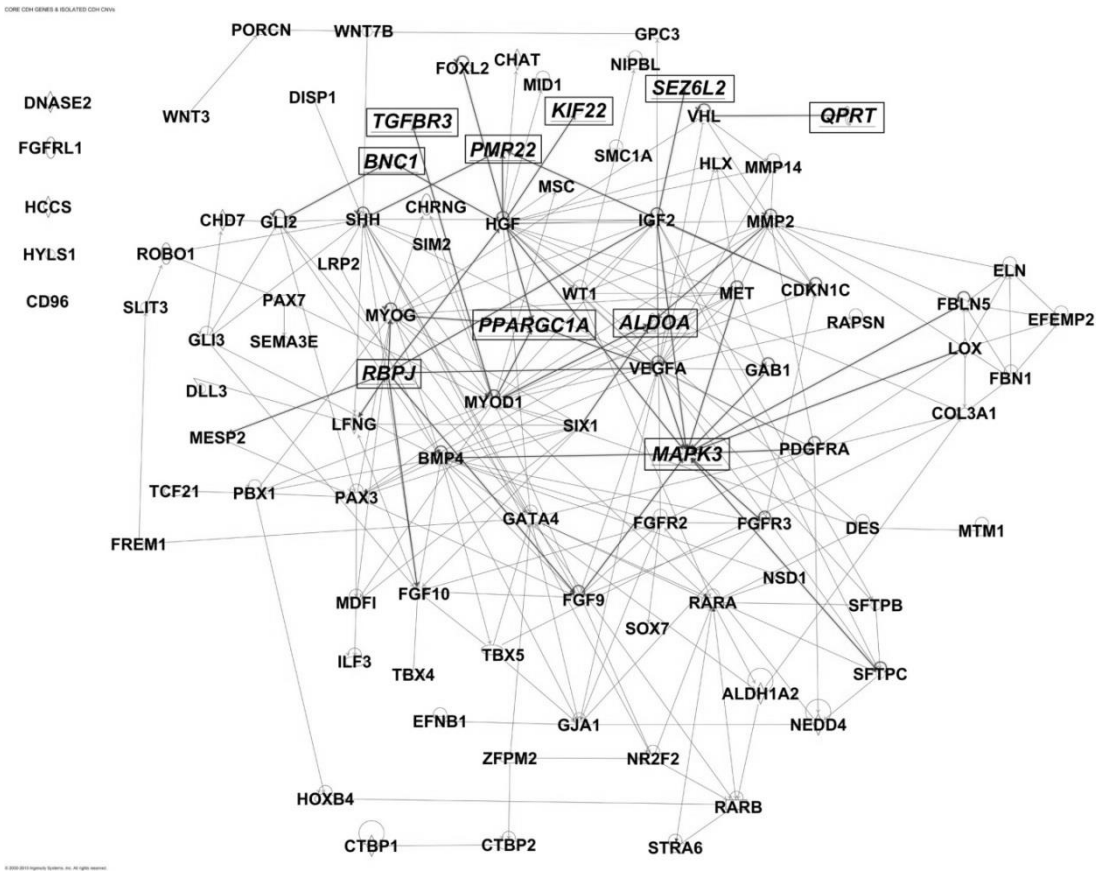

Figure 3. CDH gene network. A protein interaction network was generated using Ingenuity Pathway Analysis (IPA) and curated from; (i) a 'training gene set' (labelled in normal font); (ii) Genes within CNV loci identified in this study which connect directly to the 'training gene set' (labelled in bold italic font, in boxes). *NR2F2* and *EFNB1* are in both the 'training gene set' and present within CNVs identified in this study.

A consequence of the creation of this CDH gene network is the identification of novel candidate genes with likely involvement in CDH, but hitherto not yet identified. These include the *RBPJ* gene, present in the duplicated 4p15.2-p14 region in foetus 2, which is shown to interact with genes from the training gene set in the CDH gene network and is also ranked 1st by gene prioritisation of this CNV (online supplementary information).

Enrichment analysis was performed using the IPA software of the final CDH gene network shown in figure 3, which revealed significant enrichment for biological networks and genes known to be involved in; respiratory system development and function; cardiovascular system development and function, skeletal and muscular system development and function. These findings are concordant with observations in humans and animal models. Significant enrichment for genes involved in canonical biochemical pathways included; Regulation of the Epithelial-Mesenchymal Transition (EMT) Pathway; FGF Signalling; Axonal Guidance

Signalling; Human Embryonic Stem Cell Pluripotency; Wnt/β-catenin Signalling; and RAR Activation. These findings support candidate pathways such as the RA pathway as being involved in CDH, but also highlight pathways and genes involved with the EMT pathway, FGF signalling and Wnt/β-catenin signalling as potential candidates for involvement in human CDH. Full details of the enriched networks, and the top 20 enriched canonical pathways and the top 20 biological functions are given in online supplementary information.

Discussion

Whereas it was well known that specific microscopically visible chromosomal imbalances are associated with syndromic forms of CDH, our study shows that submicroscopic CNVs underlie a significant fraction of isolated forms as well. The identification of rare and novel CNVs has enabled the identification of dosage sensitive genes involved in CDH with *RBPJ* representing a novel candidate. We confirm the association of candidate genes such as *NR2F2* with CDH and cardiovascular malformations. In addition, we add further evidence for some genes known to cause syndromic forms of CDH, such as *EFNB1*, to also be associated with isolated CDH. This study adds to the growing body of evidence that CDH is a genetically heterogeneous disorder which can be caused by genetic variants in different developmental pathways.

Haploinsufficiency of NR2F2 causes CDH and cardiovascular malformations (CVMs)

This study identifies the smallest reported deletion of the 15q26 region in a case of CDH and coarctation of the aorta. This refines the minimal deleted region at 15q26 to a region 1.7Mb in size containing only 2 protein coding genes; *NR2F2* (also known as Coup-tfII) [OMIM *107773]; and *SPATA8* (Figure 1), and thus excludes *IGF1R* and *ARRDC4* from the previously defined minimal deleted CDH region at 15q26. The *NR2F2* gene has been considered the prime CDH candidate gene at 15q26 since tissue-specific Coup-tfII null mice were shown to develop CDH (You et al., 2005). Surprisingly, mutation analysis has not revealed pathogenic *NR2F2* variants confidently linked to CDH (Slavotinek et al., 2006; Scott et al., 2007). Little information is available for the *SPATA8* (spermatogenesis associated 8) gene, but this gene is unlikely to play a role given its identification as a testis specific expressed gene (Nie and Xiang, 2006). A recent study reported to have refined the critical region at 15q26 to a region which did not contain *NR2F2* (Mosca et al., 2011). However, the case which allowed for this refinement also had a large (50 Mb) trisomy for 2p16.3p25.3, and we suspect that the 2p duplication represents the cause of the CDH given previous reports of trisomy 2p in the

literature, including our previous study which found mosaicism for trisomy 2 in one isolated CDH foetus (Srisupundit et al., 2010; Pober, 2007; Holder et al., 2007). NR2F2 interacts with ZFPM2 (FOG2) (Huggins et al., 2001), which is also an interacting partner of GATA4 (Crispino et al., 2001), to regulate retinoic acid receptor / retinoid X receptor (RAR / RXR) dimerization and thus retinoic acid dependent gene transcription. *ZFPM2* and *GATA4* have both been implicated in CDH pathogenesis in humans and genetic mouse models (Ackerman et al., 2005; Wat et al., 2011; Jay et al., 2007; Yu et al., 2013), as well as with CVMs (Zhou et al., 2009; Crispino et al., 2001; Rajagopal et al., 2007). *NR2F2* has also been linked to cardiovascular development from studies in mouse models (Wu et al., 2013; Lin et al., 2012; Huggins et al., 2001; Pereira et al., 1999), and is proposed as a cause of CVMs in syndromic patients with deletion of 15q26 (Nakamura et al., 2011; Tumer et al., 2004; Rump et al., 2008; Davidsson et al., 2008; Poot et al., 2007). Our finding thus pinpoints haploinsufficiency of *NR2F2* as a cause of diaphragm and cardiovascular abnormalities in humans.

EFNB1 Overexpression causes CDH

The *EFNB1* gene is located on the X chromosome and mutations or deletions are associated with craniofrontonasal syndrome (CFNS) [OMIM #304110](Twigg et al., 2004; Wieland et al., 2007). CDH has previously been observed in both males & females affected with CFNS and *EFNB1* mutations (Vasudevan et al., 2006; Hogue et al., 2010). However, reports of *EFNB1* duplications are rare (Srisupundit et al., 2010; Petit et al., 2011; Babbs et al., 2011). Here, we present a mosaic ring X chromosome in a female foetus with CDH. Another foetus of a mosaic ring X chromosome identified by conventional karyotyping in association with CDH has been described (Nowaczyk et al., 1998). We have previously reported a male foetus with isolated CDH and a duplication containing only the *EFNB1* gene, which we proposed as the likely cause (Srisupundit et al., 2010). A second male with isolated CDH and duplication of Xq13.1 including *EFNB1* has since been reported (Petit et al., 2011). Taken together, this evidence adds further support for a dosage sensitive role of *EFNB1* in diaphragm development and thus represents the likely cause of CDH in this foetus. We conclude that that duplications of Xq13.1 in males, or the presence of this region on active marker X chromosomes in 45,X / 46,X, +mar(X) females, not subject to random X-inactivation or lacking the XIC, predisposes to CDH.

15q25.2 recurrent microdeletion is associated with CDH

We identified 2 cases of CDH with 15q25.2 deletions. Due to low copy repeats (LCRs) in this region, the 15q25 locus was predicted to be a hotspot for recurrent genomic rearrangements via non-allelic homologous recombination (NAHR) (Itsara et al., 2009). Whilst reports of the recurrent 15q25.2 microdeletion remain limited, there is mounting evidence that recurrent deletions of the 15q25.2 locus are associated with a significant risk of congenital diaphragmatic hernia. A recent report provides an update on patients and phenotypic features associated with deletions of this region (Doelken et al., 2013). Adding our findings to this data reveals that 6/8 reported cases of 15q25.2 recurrent microdeletions present with CDH, providing further evidence that isolated CDH represents one of the major features of the 15q25.2 recurrent microdeletion syndrome (Mefford et al., 2007; Wat et al., 2010; Yu et al., 2012). Candidate genes in this region include *BNC1* and *BTBD1. BNC1* is involved in cell proliferation (Tseng, 1998; Boldrup et al., 2012), and defective cell proliferation & migration in the pleuroperitoneal folds of the developing diaphragm have been proposed to underlie CDH (Clugston et al., 2010b). Given the musculature of the diaphragm, *BTBD1* is an interesting candidate involved in myoblast growth and differentiation (Pisani et al., 2004; Pisani et al., 2007).

16p11.2 deletions can cause isolated CDH

Deletions of 16p11.2 are a risk factor for autism. This deletion was recently reported in 2/45 cases of CDH (Wat et al., 2011). Here, we also identified a 16p11.2 deletion in a patient with CDH adding further evidence that 16p11.2 deletions may pose a risk of CDH. This combined total of 3/120 CDH cases with 16p11.2 deletions detected by array in this study and that of Wat et al (Wat et al., 2011), compared with the reported incidence of 6/22,246 in normal individuals (Rosenfeld et al., 2013), is significantly higher than expected by chance alone ($p < 0.0001$; one-tailed Chi-square test with Yates correction). Plausible candidate genes include the *TBX6* gene which is important for specification of paraxial mesoderm structures (Yasuhiko et al., 2006; Yasuhiko et al., 2008; Nowotschin et al., 2012) and also interacts with *MESP2* which is associated with spondylocostal dysostosis (SCD) (Whittock et al., 2004). A link to SCD and diaphragm abnormalities has been reported (Day and Fryer, 2003; Lam et al., 1999; Rodriguez et al., 2004), with some individuals affected with SCD and CDH having mutations in *DLL3* (Bulman et al., 2000). Both *MESP2* and *DLL3* are involved in Notch signalling, as is the *LFNG* gene which has also been associated with SCD (Sparrow et al., 2006).

Novel CNVs Associated with CDH & New Candidate Genes

We identify novel CNVs which may be responsible for CDH pathogenesis. These CNVs range in size from 13.5Mb to 100kb, encompassing 100's of genes to a single gene, and include both *de novo* and inherited CNVs.

A 13.5Mb *de novo* duplication of 4p15.2-p14 identified in a foetus with CDH contains the *RBPJ* gene which represents a novel CDH candidate. *RBPJ* is a mediator of Notch signalling and knockout mouse embryos develop severe vascular defects, as well as defective somite generation and heart looping morphogenesis (Krebs et al., 2004). *RBPJ* dependent notch signalling has also been shown to be a crucial regulator of mesenchymal progenitor cell proliferation and differentiation during skeletal development (Dong et al., 2010). *RBPJ* mutations have been linked to Adams-Oliver syndrome in humans (AOS3, OMIM #614814).

The *PPARGC1A* gene is also present in the duplicated region of 4p15.2-p14. PPARGC1A is a co-activator of PPARG, which is known to form heterodimers with retinoid X receptors (RXRs) to regulate gene transcription. PPARG has recently been shown to be downregulated in the nitrofen-induced hypoplastic lungs of the CDH rodent (Gosemann et al., 2012), and the authors propose that downregulation of PPARG and the associated upregulation of MCP-1 may impair lung maturation by disrupting surfactant production and alveolar maturation by interference with epithelial-mesenchymal interactions. In contrast, other studies have suggested that overexpression of PPARGC1A and PPARG are predicted to have protective effects on skeletal muscle atrophy and lung development (Kang and Li, 2012; Wang et al., 2009). *PPARGC1A* may thus represent a novel dosage sensitive gene involved in development or severity of CDH.

An inherited partial duplication of the *TGFBR3* gene was observed in foetus 1 referred due to apparently isolated CDH, but with clubfoot and hydrops subsequently identified. *TGFBR3* plays an essential role in coronary vasculogenesis (Compton et al., 2007), and is important for BMP-induced epithelial-mesenchymal transition (EMT) (Kirkbride et al., 2008), factors which may be relevant for PPF and diaphragm development, since it is proposed that a mesenchymal cell defect in the PPFs underlies subsequent CDH development – the mesenchymal hit hypothesis (Jay et al., 2007; Clugston et al., 2008; Jesudason, 2006; Clugston et al., 2010b).

Conclusion

Mounting evidence from array studies supports genetic variation as being a significant cause of CDH. We show that 9% (and as much as 13%) of isolated CDH patients, or those with CDH associated with cardiovascular malformations, harbour pathogenic submicroscopic CNVs associated with CDH. This diagnostic yield is higher than the ~6% rate which is observed in recent studies for the routine diagnostic use chromosomal microarrays for pregnancies with ultrasound anomalies (Wapner et al., 2012; Shaffer et al., 2012). We therefore advocate the use of chromosomal microarrays for CNV detection in the prenatal diagnosis of foetuses with isolated or non-isolated CDH. Despite the reduced penetrance of CDH, arrays have proven to be a powerful approach in identifying genes underlying both isolated and syndromic cases of CDH. Further use of chromosomal microarrays for the investigation of isolated CDH patients will continue to identify loci and genes which are involved in CDH. The combination of array analysis along with clinical outcome and long term follow up of survivors will allow for the determination of which submicroscopic CNVs are associated with a poor outcome.

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Chapter 5

Exome Sequencing to Identify the Genetic Cause of CDH

Chapter 5. Exome Sequencing to Identify the Genetic Cause of CDH

Adapted from;

Exome sequencing identifies *ZFPM2* as a cause of familial isolated Congenital Diaphragmatic Hernia and possibly cardiovascular malformations.

(Manuscript under review, European Journal of Medical Genetics).

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Expanding the phenotypic spectrum of *PORCN* mutations in two males with syndromic microphthalmia.

(Manuscript submitted, European Journal of Human Genetics).

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Exome sequencing identifies a recessive *PIGN* splice site mutation as a cause of syndromic congenital diaphragmatic hernia.

(Manuscript under review, European Journal of Medical Genetics).

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Introduction

Congenital Diaphragmatic Hernia (CDH) is a congenital abnormality affecting diaphragm and lung development with an incidence of 1.7 to 5.7 per 10,000 live-born infants (Kotecha et al., 2012). CDH occurs either as an isolated defect or in syndromic form for which the prognosis is worse. CDH is associated with variable degrees of pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality rate, and much of the morbidity observed in survivors. Survival rate of isolated CDH is in the range of 60-70% and better in high-volume centres (Kotecha et al., 2012).

The generation of genetic mouse models with diaphragm defects has identified a number of genes which cause CDH, although some models are either tissue-specific or produce hypomorphic alleles (Jay et al., 2007; Ackerman et al., 2005; Wat et al., 2012; You et al., 2005). RNA expression analysis and immunohistochemistry analysis in normal and teratogenic animal models (Clugston et al., 2010; Russell et al., 2012; Dingemann et al., 2012) has also confirmed gene expression and protein localisation of numerous candidate CDH genes during normal and/or abnormal diaphragm development. Most recently, defective development of the pleuroperitoneal folds (PPFs) is proposed as an underlying cause of subsequent CDH development. From those studies and through applying network analysis of interacting genes/proteins, some of the most important pathways that have been identified are the retinoic acid (RA), SHH, WNT and TGFβ signalling pathways (Holder et al., 2007; Kantarci and Donahoe, 2007; van Loenhout et al., 2009; Brady et al., 2010; Russell et al., 2012).

Analogous with those animal models, CDH is a key feature of numerous genetic syndromes in humans. An OMIM search for 'diaphragmatic hernia' returns > 200 genetic syndromes, genomic loci or genes linked to human CDH. Some of those more frequently associated with CDH are given in Table 1.

Mutation analysis has been performed on cohorts of isolated and non-isolated CDH patients for a limited number of candidate CDH genes, summarized in Table 2. Surprisingly however, these studies have identified only a small number of variants in those genes which are likely causal for *isolated* CDH. One potential reason for the failure to detect causative mutations in such retrospective targeted gene sequencing studies is the high genetic heterogeneity which underlies CDH.

Syndromes and genomic loci associated with CDH in humans	OMIM Syndrome/Locus	Causal or Candidate CDH gene(s)	OMIM Gene
Trisomy 18		unknown	n/a
Tetrasomy 12p Pallister Killian syndrome	#601803	unknown	n/a
15q26 deletion	%142340 [DIH1]	*NR2F2*	*107773
8p23.1 deletion	%222400 [DIH2]	*GATA4* *SOX7*	*600576 *612202
8q23	#610187 [DIH3]	*ZFPM2*	*603693
4p16.3 deletion Wolf-Hirschhorn syndrome	#194190	*FGFR3* *FGFRL1*	*134934 *605830
15q25.2 deletion		unknown	
22q11.2 deletion/duplication	#188400 / #608363	*TBX1*	*602054
Matthew Wood syndrome 15q24 microdeletion	#601186	*STRA6*	*610745
Denys-Drash syndrome	#194080	*WT1*	*607102
Donnai Barrow syndrome	#222448	*LRP2*	*600073
Cornelia de Lange syndrome	#122470 / #300590	*NIPBL* *SMC1A*	*608667 *300040
Craniofrontonasal syndrome	#304110	*EFNB1*	*300035
Fryns Syndrome (Possible locus at 1q41-42)	%229850 (#612530)	*(DISP1)*	(*607502)

Table 1. Examples of syndromes and genomic loci associated with CDH in humans, with estimates of CDH frequencies and respective candidate genes. Adapted from Holder, and Adapted from Brady review and custom array design.

locus	Gene(s)	No. of patients (no. with variants)	Mutations	Study
8q23	*ZFPM2 (Fog2)*	30 (1)	p.R112X (de novo, het)	(Ackerman et al., 2005)
8q23 4q12	*FOG2* *PDGFRA*	96 *(2)* 96 *(1)*	*p.M703L; p.T843A (het)* *p.L967V (het)* (parents unavailable)	(Bleyl et al., 2007)
15q26	*SIAT8B, CHD2, MCTP2, NR2F2, ARRDC4, RGMA*	>100 (0)	No pathogenic variants	(Slavotinek et al., 2006)
15q26	*NR2F2*	73 (0)	No pathogenic variants	(Scott et al., 2007)
1q41-42	*DISP1* *HLX*	179 (1) 179 *(1)*	p.A1471G (de novo, het) *p.F9L (mat, het)*	(Kantarci et al., 2010)
4p16.3	*FGFRL1*	54 (0)	No pathogenic variants	(Lopez Jimenez et al., 2010)
8p23.1	*GATA4* (exome seq) *GATA4*	1 (1) 96 (1)	p.R252W (pat, het) p.R283H (de novo, het)	(Yu et al., 2013)
11p13	*WT1*	27 (0)	No pathogenic variants	(Nordenskjold et al., 1996)

Table 2. Presents the results from sequencing analysis of candidate CDH genes in mixed cohorts of isolated and non-isolated CDH patients. Genes investigated, numbers of CDH patients studied, and any novel or rare mutations discovered are listed. Those mutations in red italics are of uncertain pathogenicity.

Exome sequencing is an attractive approach to the study of CDH, in the absence of any pathogenic structural rearrangement identified by cytogenetic or microarray analysis, because this would enable the interrogation of those candidate CDH genes identified by CNV analysis and from animal studies, as well as all known genes for causative mutations. One potential reason for the failure to detect causative mutations in such retrospective targeted gene sequencing studies is the high genetic heterogeneity which underlies CDH. A large degree of genetic heterogeneity is observed in syndromic cases of CDH, with many monogenic disorders having an association (Slavotinek, 2007), as well as large structural rearrangements and CNVs (Holder et al., 2007). More recently, we and others have demonstrated that submicroscopic CNVs are a cause of isolated CDH (Srisupundit et al., 2010; Wat et al., 2011), (Chapter 4), also caused by genomic loci scattered throughout the genome. The large number of genes associated with syndromic forms of CDH and the many genomic loci which share an association with both isolated and non-isolated forms, and the large number of candidate genes which have not been proven in humans, make exome sequencing an attractive approach. A similar approach has been successfully applied to larger patient cohorts for genetically heterogeneous disorders with large numbers of candidate genes including intellectual disability (ID) (de Ligt et al., 2012; Rauch et al., 2012) and congenital heart disease (Zaidi et al., 2013), identifying causal mutations in known or novel genes in a significant proportion of individuals studied. For ID and heart disease, the search was mainly for identification of highly penetrant *de novo* events. However, exome sequence analysis for causative variants responsible for CDH may be more challenging due to variable penetrance and / or expressivity of the phenotype.

We hypothesised that by applying exome sequencing to the study of CDH we would identify pathogenic variants, thus identifying (novel) genes linked to CDH in humans.

Methods

Chromosomal Microarray Analysis

Chromosomal microarray analysis was performed using the CytoSure Syndrome Plus 180k array (Oxford Gene Technology - OGT, Oxford, UK) for all affected individuals to exclude any

pathogenic CNV(s) as the cause, as previously described in Chapter 3 & 4 (Brady et al., 2013a; Brady et al., 2013b).

Targeted enrichment and exome sequencing

Genomic DNA was sheared by sonication, and whole genome sequencing libraries were prepared using the TruSeq DNA Library Preparation Kit (Illumina) in which platform-specific adaptors and unique DNA indexes were ligated. The gel-free method was performed with no size selection of fragments. DNA sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v2.0 or v3.0 (Roche, NimbleGen), and 2 × 100-bp paired-end reads were generated on a HiSeq2000 (Illumina) with 3 or 4 exome-seq samples pooled per lane of a sequencing flow-cell. Sheared DNA, whole genome libraries and enriched exome-seq libraries were validated using DNA-1000 chips on the BioAnalyser (Agilent), and library concentrations were determined using the dsDNA BR Assay on the Qubit (Invitrogen).

Mapping and variant calling

The paired-end sequence reads were aligned to the human genome (hg19) with the Burrows-Wheeler Aligner (BWA; version 0.5.9 – 0.6.2) (Li and Durbin, 2009) using default settings, and the read trimming parameter was set to 15. SAMtools (version 0.1.12a) (Li et al., 2009) was used for converting (SAM/BAM), sorting and indexing alignments. The quality metrics for mapping were calculated with Picard tools (version 1.38 – 1.78). Duplicate reads were marked with Picard tools and excluded from downstream analysis. The GATK framework (version 1.0.4974 – 2.4.9) (McKenna et al., 2010) was used for performing the local realignment, base call recalibration and SNP calling. Indels were called with Dindel (version 1.01) (Albers et al., 2011) using default parameters. Variants were annotated with ANNOVAR (version 2011 – version 2013) (Wang et al., 2010), which provides information including dbSNP (dbSNP132 – dbSNP137), and 1000 Genomes data (release May 2011 – release April 2012). The current analysis pipeline includes annotation for the ESP6500 panel (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA), (http://evs.gs.washington.edu/EVS/). The current exome variant server (EVS) data release (ESP6500SI-v2) is taken from 6503 samples drawn from multiple ESP cohorts and represents all of the ESP exome variant data. Functional predictions for the amino-acid changes according to different models is also provided (SIFT, Polyphen2, LRT and MutationTaster)

were retrieved from dbNSFP (database of human nonsynonymous SNPs and their functional predictions) (Liu et al., 2011).

Variant Filtering

Variant files annotated by the GATK analysis pipeline were filtered against provided annotations in Excel, and using the web application 'Annotate-it' (Sifrim et al., 2012) (http://www.annotate-it.org/). Details of filters applied by each method and the number of variants remaining after filtering are provided in the results section. Annotate-it provides details at the gene level for pathways and biological processes associated with the respective gene function. Additionally, annotate-it provides text-based genotype-phenotype associations (according to HPO and LDDB terms) from 'A Gene Apart' and respective p-values for this association. Variants of interest were also checked against the Exome Variant Server, (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA), (http://evs.gs.washington.edu/EVS/). The current in-house analysis pipeline now provides annotation for the ESP 6500 dataset in the variant files.

Variant confirmation

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/), and SpanPrimers (http://medgen.ua.ac.be/~gvandeweyer/SpanPrimers/index.php). PCR products were purified with ExoSAP-IT (GE Healthcare) and sequenced using BigDye Terminator v3.1 chemistry (Life Technologies) on a 3730 DNA Analyzer (Life Technologies). Sequence traces were aligned to the reference sequence using Sequence Scanner v1.0 software (Applied Biosystems) and CLC Main Workbench v6 (CLC Bio).

X-Inactivation Analysis

The methylation status of the X-linked androgen receptor (AR) gene was assessed by gene methylation assay, as previously described (Allen et al., 1992; Froyen et al., 2007). The resulting products were separated on an ABI 3730 (Applied Biosystems) and peak positions and peak intensity areas were further processed using Excel to calculate the percentage inactivation of both alleles.

Patients

Written, informed consent was provided for exome sequencing by patients prior to analysis. Details of the phenotypes are described below, per family, and the family pedigrees are shown in Figure 2.

Family 1

The parents (II.1 and II.2) have a total of 4 pregnancies; the family pedigree is shown in Figure 2. The first child (III.1) is a healthy male; the second pregnancy (III.2) was a female foetus, with TOP at 5 months due to apparently isolated CDH; the third pregnancy (III.3) was a male foetus, with TOP at 5 months due to apparently isolated CDH, confirmed at autopsy examination; the fourth pregnancy (III.4) led to a male child, with a cardiac defect [total anomalous pulmonary venous return (TAPVR)] detected neonatally, with subsequent corrective surgery, and no current health problems. Upon a later X-ray, this child was shown to have a right-sided anterior diaphragm eventration, which has no apparent effects on respiration or health. Exome sequencing was undertaken for both parents (II.1 and II.2) and for the 2 foetuses affected with isolated CDH (III.2 and III.3).

Family 2

From the first relationship of the mother (I.2), with partner (I.3), two healthy daughters were born (II.5 and II.6). From her current relationship there were two affected male foetuses (II.1 and II.2) and two healthy daughters (II.3 and II.4), see pedigree, Figure 2. Patient II.1 was born with microphthalmia and coloboma of the retina, a left-sided posterolateral diaphragmatic hernia, and an atrial septal defect (ASD). He was deceased at day 0, with confirmation of the clinical findings by pathology examination. Patient II.2 had multiple anomalies that were detected at 28 weeks of gestation and manifest as bilateral microphthalmia with dens intra-ocular tissue, a large thoraco-lumbar spina bifida and hydronephrosis of the left kidney. He was born at 38 weeks of gestation, with hypospadias and disarrayed toes detected postnatally, and he was deceased after 10 days due to respiratory insufficiency. No post-mortem examination was performed. A potential diagnosis of MCOPS9 or Matthew Wood syndrome [OMIM #601186] was made, but mutation analysis of the *STRA6* gene was negative for any causal variants in patient II.2 (performed at the Institute for Human Genetics, Erlangen, Nurnberg, Germany). Exome sequencing was undertaken for both affected foetuses (II.1 and II.2) and the parents (I.1 and I.2).

Family 3

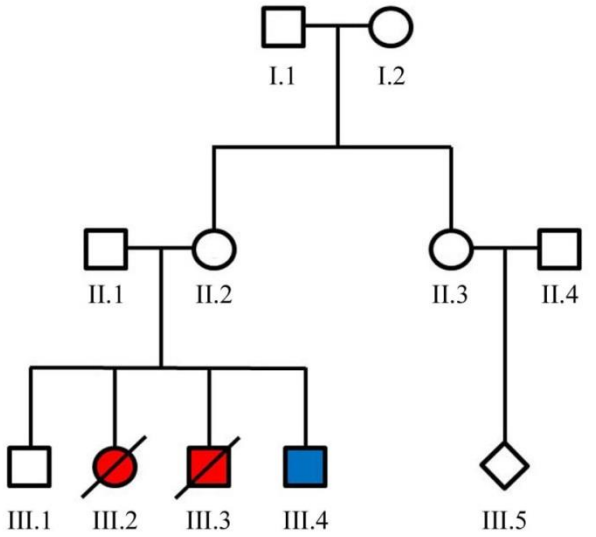
The index is a foetus, the first pregnancy of consanguineous parents (first cousins, once removed), of North-African descent. Pregnancy was terminated at a gestational age of 16 weeks because of a diaphragmatic hernia and associated malformations. At autopsy, facial

dysmorphism was observed (figure 1a & b); a broad nose, hypertelorism, anteversion of the nostrils and low set, dysplastic ears. There was a hygroma colli and mild axillary pterygia, a cleft palate, and small penis with hypospadias. The left foot (figure 1c) was abnormal with oligodactyly (absence of rays 3-5), and hypoplasia of the remaining toes; the first toe had no nail and lacked a proximal phalanx, the second ray lacked a nail, and the middle phalanx was missing. Attached to the left heel, there was a synovial cyst (figure 1a and c). The right foot appeared normal. Internal examination confirmed the bilateral postero-lateral diaphragmatic hernia. There were cardiovascular anomalies, a muscular VSD, overriding aorta, hypoplastic pulmonary trunk, an aberrant retro-esophageal right subclavia (lusoria). There was gut malrotation, cryptorchidism, and the kidneys revealed segmental renal dysplasia. Images of the foetus are shown in Figure 1 (A-C). The affected foetus (II.1) and both parents (I.1 and I.2) underwent exome sequencing.

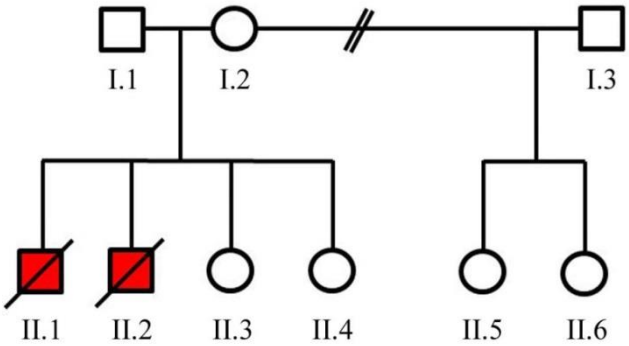
Figure 1. Images of the affected foetus. A. Full body view, from right. B. Frontal view showing head and chest. C. Close-up of left foot.

Pedigrees

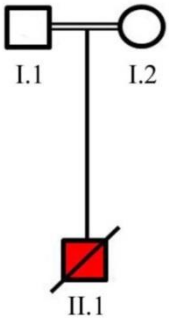
Figure 2. Pedigrees for Family 1; Family 2; and Family 3.



Family 1.



Family 2.



Family 3.

Results

Exome Sequencing Quality Metrics

The tables below (Table 3; Table 4; and Table5) provide quality metrics for a number of key parameters provided by Picard metrics (http://picard.sourceforge.net/index.shtml). The associated graphs, shown in Figure 3, provide % target bases covered at 2x, 10x, 20x, and 30x coverage.

Patient	Total Reads (number)	Aligned Reads (number)	Aligned Reads (%)	Mean Coverage of Target Region	Target Fold Enrichment	Targets with Zero Coverage (%)
III.2	60329403	59631806	98.8	72	38	2.2
III.3	55295963	54638871	98.8	65	38	2.2
II.1	76908967	75873223	98.7	91	39	1.8
II.2	67560828	66679281	98.7	77	37	2.1

Table 3; Exome Sequencing Quality Metrics for Family 1.

Patient	Total Reads (number)	Aligned Reads (number)	Aligned Reads (%)	Mean Coverage of Target Region	Target Fold Enrichment	Targets with Zero Coverage (%)
I.2	52744438	50996671	96.7	39	22	13
I.1	64301594	60844058	94.6	42	20	11
II.1	65003977	62669610	96.4	43	20	11
II.2	59010896	56759670	96.2	36	19	10

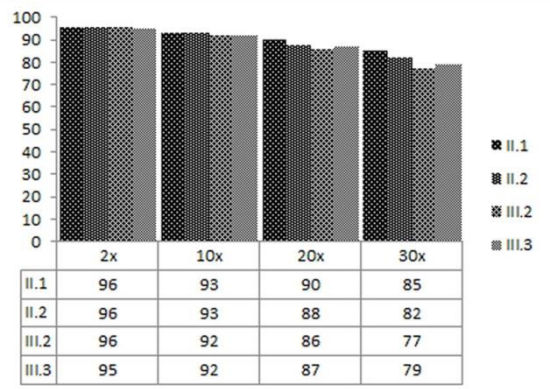
Table 4; Exome Sequencing Quality Metrics for Family 2.

Patient	Total Reads (number)	Aligned Reads (number)	Aligned Reads (%)	Mean Coverage of Target Region	Target Fold Enrichment	Targets with Zero Coverage (%)
I.1	82405961	82083567	99.6	50	21	2.1
I.2	79009381	78746637	99.7	49	21	2.4
II.1	85315952	85012247	99.6	52	21	2.2

Table 5; Exome Sequencing Quality Metrics for Family 3.

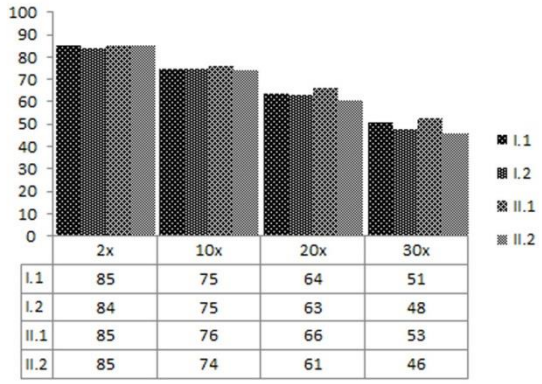
Tables 3, 4, 5. The tables above provide metric details for; Total Read number; Aligned Reads number; % Reads Aligned; Mean Target Coverage; Target Fold Enrichment; % Targets with Zero Coverage. Details are provided per individual and displayed by family.

Family 1.



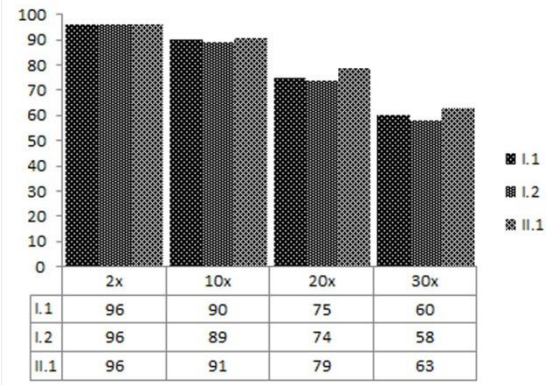
	2x	10x	20x	30x
II.1	96	93	90	85
II.2	96	93	88	82
III.2	96	92	86	77
III.3	95	92	87	79

Family 2.



	2x	10x	20x	30x
I.1	85	75	64	51
I.2	84	75	63	48
II.1	85	76	66	53
II.2	85	74	61	46

Family 3.



	2x	10x	20x	30x
I.1	96	90	75	60
I.2	96	89	74	58
II.1	96	91	79	63

Figure 3. The graphs above provide values for the percentage of target bases covered at 2x, 10x, 20x, and 30x coverage. Values are provided per individual and displayed by family.

Results: Family 1

Retrospective chromosomal microarray analysis of fetal material from both affected foetuses revealed no pathogenic CNVs. With two affected foetuses with isolated CDH a familial cause was suspected. We therefore applied exome sequencing in the two affected foetuses (III.2 and III.3) and the parents (II.1 and II.2) to search for inherited variants which could explain the cause of recurrent CDH in this family.

Exome-seq and Variant Filtering Strategy.

Variant filtering using Excel for a possible recessive cause (i.e. homozygous or compound heterozygote variants in the affected individuals) identified no obviously pathogenic candidate variants when filtering against dbSNP. However, when filtered against variants present in the 1000genomes database rather than dbSNP, a candidate pathogenic variant was identified in both affected individuals (III.2 and III.3). Details of the parameters used are provided in Table 6 and Table 7. Functional predictions were used to filter for damaging non-synonymous variants. Variant filtering identified a stop (nonsense) mutation in exon 4 of *ZFPM2* [NM_012082: ENST00000407775: c.C334T: p.Arg112*]. Since this mutation creates a premature stop codon the identification of this variant was aided by manual filtering of stop-gain or –loss, and splice site mutations against a list of candidate CDH genes. 'Annotate-it' (Sifrim et al., 2012) was also used for variant filtering to search for compound heterozygote variants, but did not reveal any apparently plausible candidate variants. Using criteria to search for rare variants in both affected individuals and inherited from one of the parents, the same *ZFPM2* p.Arg112* variant was identified by Annotate-it. Again, identification of the candidate variant was aided by filtering against a list of candidate CDH genes. The ability to combine filtering strategies in annotate-it across multiple samples created far more manageable candidate variant lists from which to identify the potential pathogenic variant. The candidate variant details are provided in Table 8.

			III.2	III.3
		Total number of Variants	**41836**	**40100**
	Quality Filter	Depth > 10 Q (phred) > 30	31733	31035
Variant Type	Non-synonymous	total Not dbSNP Not 1000g Rare Variant <1% <1% or absent 1000g	10756 761 1184 252 (1436 combined)	10698 677 1106 243 (1349 combined)
	Stop Gain	Total Not dbSNP	**130** 12	**103** 10
	Stop Loss	Total Not dbSNP	24 1	26 0
	Exonic Splicing	Total Not dbSNP	395 14	389 17

Table 6. Excel Variant Filtering

		Rare Paternal Variants	**Rare Maternal Variants**	**Compound Heterozygote**
Included Samples		III.2, III.3, II.1(pat) In at least 3 samples	III.2, III.3, II.2(mat)	III.2, III.3 compound heterozygote
Excluded Samples		II.2(mat)	II.1(pat)	None
Filter Included Samples		Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%
Filter Excluded Samples		Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%	None
Variant Type	Nonsense Splice Site Non-synonymous Synonymous UTR	Yes Yes Yes No No	Yes Yes Yes No No	Yes Yes Yes No No
dbSNP		Not used	Not used	Not used
1000genomes	Population Frequency	< 1%	< 1%	< 1%
200 Danish Exomes		Not used	Not used	Not used
Prediction Criteria	SIFT Polyphen2 LRT Mutation Taster	NO [YES]	NO [YES]	NO [YES]
Total number of Variants		58 [5]	**63** [4]	11 [0]

Table 7. Annotate-it Variant Filtering

	III.2	III.3	II.1 (Father)	II.2 (Mother)
Chromosome	8	8	8	8
Position	106573623	106573623	106573623	106573623
Gene Name	ZFPM2	ZFPM2	ZFPM2	ZFPM2
Depth	40	26	28	32
Reference Allele	C	C	C	C
Number of reads with Ref. allele [freq.]	25 [0.62]	15 [0.57]	28 [1.0]	11 [0.34]
Alternate Allele	T	T	-	T
Number of reads with Alt. allele [freq.]	15 [0.37]	11 [0.42]	-	21 [0.65]
Mutation Type	Nonsense	Nonsense	-	Nonsense
Refseq accession ID	NM_012082	NM_012082	-	NM_012082
Mutation DNA	c.C334T	c.C334T	-	c.C334T
Mutation Protein	p.Arg112*	p.Arg112*	-	p.Arg112*
Functional Predictions; - SIFT - Polyphen2 - LRT - Mutation Taster	None	None	-	None
Confirmed by Sanger Seq	Yes	Yes	Yes	Yes
dbSNP132 – dbSNP137	rs121908602 probable pathogenic allele	rs121908602 probable pathogenic allele		rs121908602 probable pathogenic allele

Table 8. Variant Details for Main Candidate

In order to exclude germline mosaicism as a contributory factor, de novo variants were searched for. This did not reveal any apparent de novo events which were shared by both affected individuals. To exclude that a second causal variant in ZFPM2 was removed by the variant filtering process or had no read coverage, the .bam files were manually checked in a genome browser for each exon of ZFPM2 for each individual. All exons of ZFPM2 are covered sufficiently (>15x coverage) with no additional mutation(s) observed, except for the first 9 and 12 bases of exon 1, covered at 5x and 4x respectively in the 2 affected individuals. None of those reads contained any ZFPM2 variant in those bases with low coverage in any individual. The full list of rare variants for both affected individuals is provided in supplementary tables S3 and S4 (depth>10; Q>30; population frequency <0.03; and coding).

Familial Analysis

Mutation analysis for the p.Arg112* mutation by conventional Sanger sequencing confirmed the presence of the mutation in both siblings (III.2 and III.3) and the mother (II.2). In addition, other family members (III.4, I.1, and I.2) were subsequently tested for the presence of this variant, shown in Figure 4. The sibling with a TAPVR (III.4), who was also shown to

have a mild diaphragmatic eventration, was confirmed to have inherited the same maternal p.Arg112* mutation. Images of the diaphragm eventration which was visible on X-ray after surgical correction of the TAPVR are shown in Figure 5. In order to determine if the variant had occurred *de novo* in the mother (II.2) those grandparents (I.1 and I.2) were tested which revealed the variant was inherited from the maternal grandfather (I.1). The maternal sister (II.3) was subsequently tested, which was also positive for the same variant. The mother of the affected foetuses (II.2), her father (I.1) and her sister (II.3) all carry the *ZFPM2* p.Arg112* variant, but are all asymptomatic with no diaphragm or heart abnormalities reported in any of these individuals. Individuals III.1, II.4, and foetus III.5 were not tested.

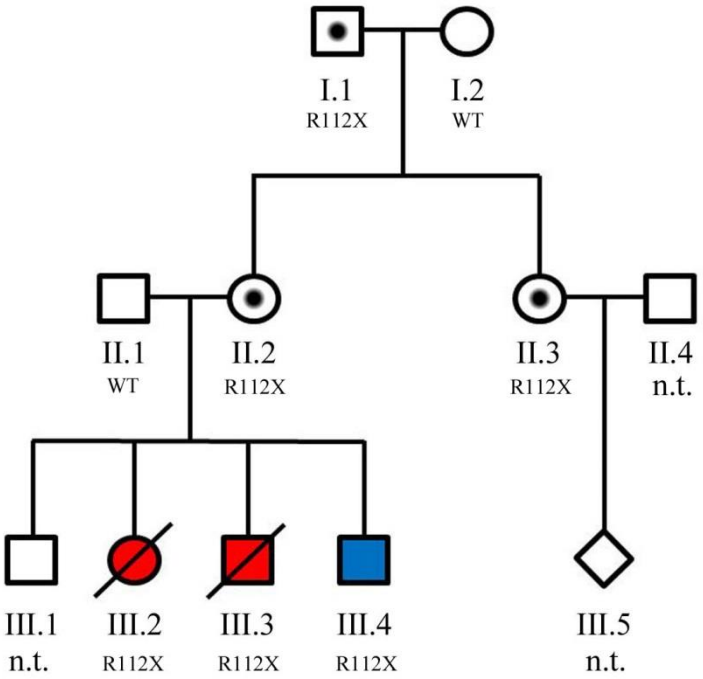


Figure 4. Family 1 Pedigree; Individuals II.1, II.2, III.2, and III.3 underwent exome-sequencing which revealed the R112X mutation in the *ZFPM2* gene in both affected individuals (III.2 and III.3) as well the asymptomatic mother (II.2). Further analysis of additional family members revealed that the sibling III.4, affected with a TAPVR also shares the mutation which is likely to be responsible for the heart defect. The grandfather (I.1), and the maternal sister (II.3), both of whom are asymptomatic also carry the mutation. Individuals III.1, II.4, and foetus III.5 were not tested (n.t.). WT = wild type allele (II.1 and I.2).

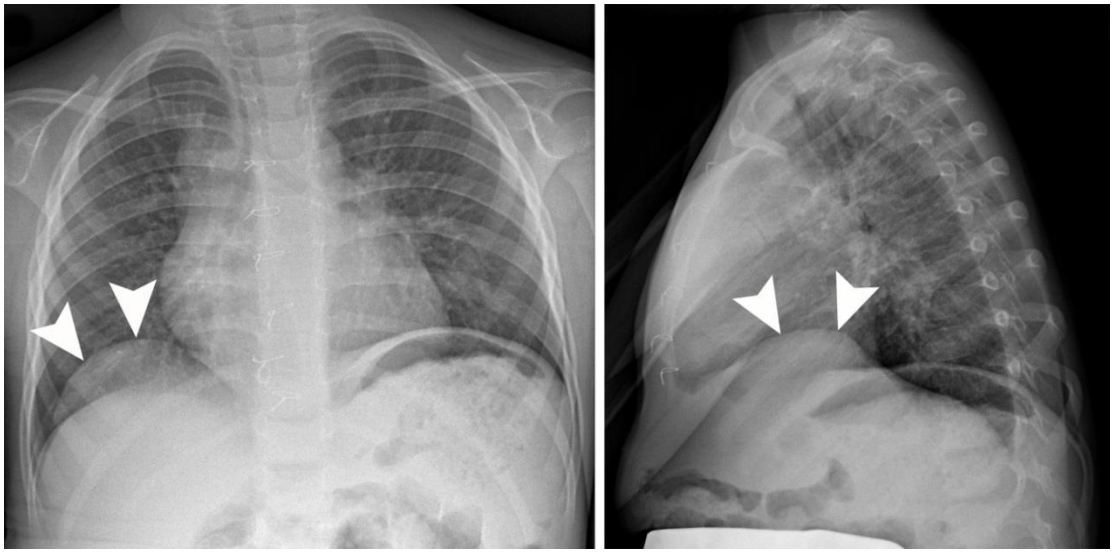


Figure 5. Shows the diaphragm eventration observed on X-ray in the sibling affected with a total anomalous pulmonary venous return (TAPVR) who was shown to also carry the p.Arg112* *ZFPM2* mutation. The left image shows the frontal view and the right image shows the view from the patients left side. The anterior right sided diaphragm eventration is highlighted with the white arrow heads.

Discussion: Family 1

We undertook exome sequencing in a familial case of isolated CDH which identified a heterozygous nonsense mutation (p.Arg112*) in the *ZFPM2* (or *FOG2*) gene in 2 foetuses affected with isolated CDH. The *ZFPM2* p.Arg112* mutation has a dbSNP entry (rs121908602) and is therefore removed when filtering against dbSNP variants (from dbSNP132 onwards). In dbSNP this 'SNP' is annotated as a pathogenic allele, albeit observed in only one individual in association with a diaphragmatic eventration (Ackerman et al., 2005). The *ZFPM2* gene has been previously investigated for a role in CDH following identification of diaphragm and primary lung abnormalities in an ENU-derived mouse model (Ackerman et al., 2005). The authors subsequently sequenced the *ZFPM2* gene in 30 (deceased) CDH patients and reported a heterozygous nonsense mutation, shown to have occurred *de novo*, in a patient who died at birth with severe pulmonary hypoplasia and a posterior diaphragmatic eventration (Ackerman et al., 2005). Interestingly, it is the exact same p.Arg112* variant which we identify in this study. Bleyl et al sequenced the ZFPM2 gene in an additional 96 individuals with CDH and identified novel sequence alterations predicting p.M703L and p.T843A in two patients with isolated CDH. However, due to the lack of parental DNA samples the authors were not able to determine if the sequence alterations were de novo (Bleyl et al., 2007). In order to determine the potential functional effects of those mutations, we manually checked the Exome Variant Server (EVS) database

for their presence in normal individuals, and SIFT and PolyPhen-2 functionally damaging predictions were calculated. The M703L mutation is not present in the EVS database, is predicted tolerated by SIFT, and identified as possibly damaging (HumDiv) or probably damaging (HumVar) by Polyphen-2. The T843A mutation is also not present in the EVS database, is also predicted tolerated by SIFT, and possibly damaging (HumDiv) or benign (HumVar) by Polyphen-2. *ZFPM2* mutation analysis alone is thus somewhat inconclusive as a cause of isolated CDH, with only a single deleterious *de novo* mutation so far associated with a diaphragmatic eventration (Ackerman et al., 2005).

Evidence from chromosomal microarray analysis of CDH patients however does add further support for pathogenicity. Chromosomal microarray analysis revealed submicroscopic copy number variations (CNVs) causing (partial) deletion of ZFPM2 considered causal for CDH, but which were transmitted from an apparently unaffected parent (Wat et al., 2011). The authors propose that haploinsufficiency of ZFPM2 can cause dominantly inherited isolated diaphragmatic defects with incomplete penetrance. Kuechler et al have previously described 5 patients with overlapping deletions of 8q22q23 (Kuechler et al., 2011). In 2 of these patients the *ZFPM2* was partially deleted; one had CDH and the other had a hiatal hernia. There are no nonsense, splicing or frameshift mutations in ZFPM2 reported in the exome variant server database, and no CNVs exist in the database of genomic variants (DGV) of whole gene deletions of ZFPM2 in normal individuals.

Taken together, the previous finding of the identical mutation in a foetus with diaphragmatic eventration, evidence from the *ZFPM2* (*Fog2*) ENU mouse model of CDH and pulmonary hypoplasia, and supporting evidence from chromosomal microarray analysis in patients with CDH, this variant is likely to be the cause of the isolated CDH (and diaphragm eventration) in those affected in this family. This finding thus represents the first inherited *ZFPM2* mutation associated with recurrent isolated CDH in a single family and adds further evidence for haploinsufficiency of ZFPM2 as a cause of CDH. The presence in one family member (III.4), with a heart defect and mild diaphragm eventration, as well as further family members who are apparently asymptomatic (I.1, II.2, and II.3) reinforces the considerable complexity associated with CDH penetrance. This mutation displays apparent autosomal dominant inheritance with variable penetrance.

The previous observations of heart defects in association with *ZFPM2* variants suggests that the p.Arg112* variant present in individual III.4 may also be responsible for the TAPVR in this child. Heterozygous missense mutations of the *ZFPM2* gene have been reported in patients

with sporadic tetralogy of Fallot (TOF) (Pizzuti et al., 2003). Similar to Tetralogy of Fallot (TOF), double outlet right ventricle (DORV) is a conotruncal heart defect (CTD) and is anatomically characterized by a malposition of the great arteries. Sequencing of ZFPM2 has also revealed novel rare variants in patients with sporadic DORV, TOF, and/or transposition of the great arteries (Tan et al., 2012). To date, there is no definitive correlation with TAPVR, but it is plausible that this is not incidental. Interestingly, Yu et al recently applied whole exome sequencing (WES) to identify a paternally inherited novel missense GATA4 variant (c.754C>T; p.R252W) in a familial case of CDH which was also associated with incomplete penetrance. Detailed phenotypic characterization of additional family members by MRI of the chest and abdomen revealed asymptomatic defects in the diaphragm of the two "unaffected" missense variant carriers (Yu et al., 2012).

ZFPM2 interacts with GATA4 and both are considered essential for diaphragm development as well as pulmonary development and heart development from multiple studies in animal models (Molkentin et al., 1997; Crispino et al., 2001; Ackerman et al., 2005; Ackerman et al., 2007; Zhou et al., 2009; Jay et al., 2007). The RA pathway is illustrated in Figure 1.2.4, (Chapter 1, this thesis), including the involvement of ZFPM2 (Fog2), GATA4 and NR2F2 in regulation of RA dependent gene transcription. SUMO modifications are post-translational processes which regulate the biological activity of many proteins by the addition of a small ubiquitin-related modifier (SUMO) protein to target lysine residues. It has been shown recently that the biological activity of ZFPM2 is dependent on intact SUMOylation sites, with *ZFPM2* (Fog2) SUMO mutants displaying stronger transcriptional repression and more efficient interaction with GATA4 (Perdomo et al., 2012). Since SUMOylation is a dynamic and reversible process, it provides a potential mechanism for rapid fine-tuning of ZFPM2 activity, which if affected may increase the risk of CDH development in susceptible individuals.

Genetic counseling in this family is challenging; there are at least three persons in this family who are carriers of this mutation and who have no manifestations. This is known for other dominant conditions and thus probably points to additional genetic and / or environmental influences, for example, variants inherited from the other parent or epigenetic factors. To explore the potential presence of modifier variants in genes implicated to interact with ZFPM2, exome sequences were searched for rare paternal variants, including *ZFPM2* interacting genes involved in RA pathway; *NR2F2* and *GATA4*. This manual search revealed no candidate variant identifiable as a ‘second-hit’ inherited from the father. The finding of 3

asymptomatic family members clearly demonstrates that haploinsufficiency of *ZFPM2* alone is not necessary, but can be sufficient to cause CDH in combination with as yet unidentified (epi-)genetic and/or environmental influences.

Results: Family 2

Chromosomal microarray analysis of fetal material from both affected foetuses revealed no pathogenic CNVs. A clinical diagnosis of Matthew-Wood syndrome was excluded in the second affected child by mutation analysis of the *STRA6* gene by conventional Sanger sequencing. Given the high likelihood of a recessive or X-linked mode of inheritance as the cause of the severe phenotypic features, we applied exome sequencing in the 2 affected foetuses (II.1 and II.2), as well as both parents (I.1 and I.2).

Exome-seq and Variant Filtering Strategy

Variants were filtered on quality parameters, variant type and against the annotated population databases using Excel and annotate-it as shown in Table 9 and Table 10. Filtering was performed in Excel for the two affected individuals (II.1 and II.2) for possible mechanisms of inheritance (i.e. homozygous or compound heterozygote variants, or possible X-linked inheritance). Variant filtering was aided by filtering against variants present in dbSNP, the 1000 genomes database, and by using functional predictions (provided from SIFT, Polyphen2, and Mutation Taster). A shortlist of only around 30 non-synonymous variants remained in each affected individual (II.1 and II.2) which contained a strong candidate variant; a missense mutation in the PORCN gene [NM_203474: ENST00000326194: c.G470A:p.G157D. Annotate-it was used for variant filtering to search for compound heterozygote variants, but of 11 compound heterozygote variants shared by both affected individuals, none were predicted functionally damaging. Using criteria to search for rare variants in both affected individuals and inherited from one of the parents, the *PORCN* G157D variant was identified as a strong candidate. Annotate-it provides text-based genotype-phenotype associations (according to HPO and LDDB terms) from 'A Gene Apart' and respective p-values for this association. Both anophthalmia (p-value (1.348 E-05) and congenital hernia of diaphragm (p-value 7.655 E-03) are listed in association with the candidate variant in *PORCN* thus aiding the candidate variant identification process in this situation. The details for the candidate variant are shown in Table 11.

			II.1	II.2
		Total number of Variants	**52226**	**52169**
	Quality Filter	Depth > 10 Q (phred) > 30	31361	30538
Variant Type	Non-synonymous	total	10696	10449
		Not dbSNP	660	670
		Not 1000g	1084	1106
		Rare Variant <1% 1000g	241	242
		1000g combined	(1325 combined)	(1348 combined)
		dbSNP & 1000g	412	438
		SIFT	160	147
		PolyPhen	60	61
		Mutation Taster	77	69
		Predictions combined	(**28** combined)	(**32** combined)
	Stop Gain	Total	111	112
		Not dbSNP		18
	Stop Loss	Total	27	25
		Not dbSNP		0
	Exonic Splicing	Total	404	380
		Not dbSNP		10

Table 9. Excel Variant Filtering

		Rare Paternal Variants	**Rare Maternal Variants**	**Compound Heterozygote**
Included Samples		II.1, II.2, I.1(pat) In at least 3 samples	II.1, II.2, I.2(mat)	II.1, II.2 Genes must be compound heterozygote
Excluded Samples		I.2(mat)	I.1(pat)	None
Filter Included Samples		Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%
Filter Excluded Samples		Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%	None
Variant Type	Nonsense	Yes	Yes	Yes
	Splice Site	Yes	Yes	Yes
	Non-synonymous	Yes	Yes	Yes
	Synonymous	No	No	No
	UTR	No	No	No
dbSNP		Not used	Not used	Not used
1000genomes	Population Frequency	< 1%	< 1%	< 1%
200 Danish Exomes		Not used	Not used	Not used
Prediction Criteria All 4 used / None used	SIFT Polyphen2 LRT Mutation Taster	NO [YES]	NO [YES]	NO [YES]
Total number of Variants		49 [5]	**60 [7]**	11 [0]

Table 10. Annotate-it Variant Filtering

	II.1	II.2	I.1 (Father)	I.2 (Mother)
Chromosome	X	X	X	X
Position	48370810	48370810	48370810	48370810
Gene Name	PORCN	PORCN	PORCN	PORCN
Depth	57	37	37	73
Reference Allele	G	G	G	G
Number of reads with Ref. allele [freq.]	0 [0]	0 [0]	37 [1.0]	42 [0.57]
Alternate Allele	A	A	-	A
Number of reads with Alt. allele [freq.]	57 [1.0]	37 [1.0]	-	31 [0.42]
Mutation Type	nonsynonymous	nonsynonymous	-	nonsynonymous
Refseq accession ID	NM_203474	NM_203474	-	NM_203474
Mutation DNA	c.G470A	c.G470A	-	c.G470A
Mutation Protein	p.G157D	p.G157D	-	p.G157D
Functional Prediction - SIFT - Polyphen2 - LRT - Mutation Taster	 Damaging Probably Damaging Deleterious Disease Causing	 Damaging Probably Damaging Deleterious Disease Causing	-	 Damaging Probably Damaging Deleterious Disease Causing
Confirmed by Sanger Seq	Yes	Yes	Yes	Yes

Table 11. Variant Details for Main Candidate

Familial Analysis & X-inactivation analysis.

Mutations in *PORCN* are associated with Goltz-Gorlin syndrome Focal Dermal Hypoplasia (FDH; OMIM #305600), an X-linked dominant disorder in which heterozygous females are expected to be affected. Conventional Sanger sequencing of the *PORCN* gene confirmed the presence of the *PORCN* G157D mutation in the mother (I.2) and both affected males (II.1 and II.2). Analysis of the 4 daughters revealed the same mutation to be present in II.3 and II.4, but not in II.5 and II.6. We therefore investigated the X-inactivation status in genomic DNA from the mother (I.2) and the 4 daughters from both relationships (II.3 and II.4; and II.5 and II.6). This revealed extreme skewing of X-inactivation (90%/10%) in the heterozygous mother; and levels of 88%/12% and 70%/30% in the 2 heterozygous daughters (II.3 and II.4, respectively) for the same maternally inherited X chromosome. One of the heterozygous female carriers, initially considered unaffected (II.4), displayed aplasia cutis on the scalp, shown in Figure 6. The 2 daughters who do not carry the mutation (II.5 and II.6) showed random X-inactivation with levels of 54%/46% and 67%/33%, respectively and inheritance of the alternate maternal X chromosome. The pedigree is shown in Figure 7, including the results of the X-inactivation analysis.

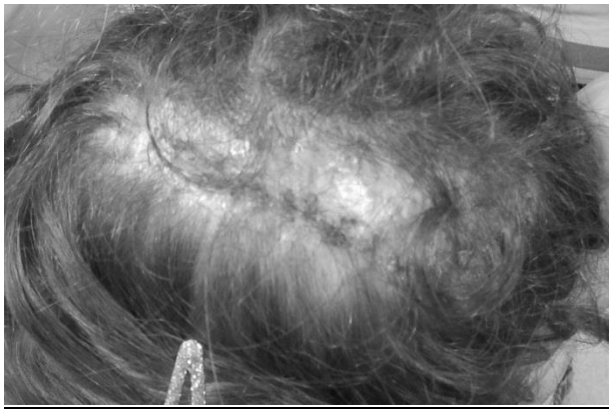


Figure 6. Cutis aplasia of the scalp in female II.4.

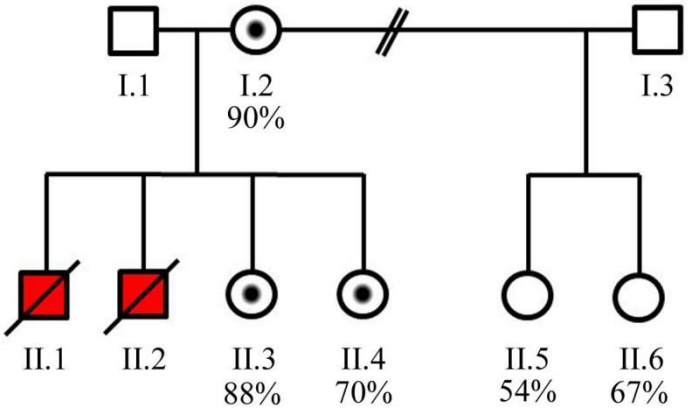


Figure 7. Family 2 Pedigree; Individuals I.1, II.2, II.1, and II.2 underwent exome-sequencing which revealed the G157D mutation in the *PORCN* gene in both affected individuals (III.2 and III.3) as well the asymptomatic mother (I.2) who was shown to have 90% skewing of X-inactivation. Further analysis of additional family members revealed that the two female siblings II.3 and II.4 also carry the mutation, with 88% and 70% skewing of X-inactivation, respectively. The two females from the previous relationship (II.5 and II.6) have not inherited the mutation and demonstrate 54% and 67% skewing of X-inactivation, respectively. Individual I.3 was not tested.

Discussion: Family 2

We describe the first case of non-mosaic males affected with syndromic microphthalmia due to a non-synonymous mutation in the PORCN gene. The 2 foetuses were affected with multiple congenital anomalies including; microphthalmia, CDH and an ASD (II.1); and bilateral microphthalmia and spina bifida (II.2). To our knowledge, there are 4 previous cases reported of a PORCN mutation in association with syndromic CDH; a female foetus with multiple congenital anomalies including CDH, limb anomalies, microphthalmia, and lung

anomalies; a female with phenotypic features consistent with FDH and Pentalogy of Cantrell, including an anterior diaphragmatic hernia; and 2 unrelated female foetuses born to affected mothers who displayed ectopia cordis, diaphragmatic hernia and abdominal wall defects (Maas et al., 2009; Smigiel et al., 2011; Dias et al., 2011). Our finding thus adds further support for PORCN mutations as a cause of syndromic CDH and not a coincidental association.

Mutations in *PORCN* were first reported as a cause of Focal Dermal Hypoplasia in 2007 (Wang et al., 2007; Grzeschik et al., 2007), and Froyen et al later identified *PORCN* mutations and deletions in a cohort of patients with a clinical diagnosis of FDH (Froyen et al., 2009). FDH is characterized by phenotypic features including; longitudinal striation of the long bones, the combination of split hand with syndactyly and absence of rays (also termed 'lobster-claw hand'), as well as atrophy and linear pigmentation of the skin, herniation of fat through dermal defects, and multiple papillomas of the mucous membranes or skin. Oral anomalies, in addition to lip papillomas, include hypoplastic teeth, and ocular anomalies including coloboma of iris and choroid, strabismus, and microphthalmia have also been reported. Mental retardation occurs in many of the patients. Recently, a 'mutation update' for PORCN reported a number of new cases and reviewed those reported to date (Lombardi et al., 2011).

Interestingly, FDH is observed in heterozygous females, whereas the few reports in affected males are limited to cases of somatic mosaic. FDH due to PORCN mutations are thus an X-linked dominant condition which, until now, were considered to result in in utero lethality in males. Our finding thus represents the first report of affected non-mosaic males, adding evidence for PORCN mutations in males as a cause of phenotypic features including eye anomalies, diaphragm defects, spina bifida, cardiac defects, kidney defects and syndactyly / polydactyly. This is in contrast to some of the characteristic phenotypic features more commonly seen in females affected with Goltz-Gorlin syndrome or focal dermal hypoplasia. The possible clinical diagnosis of Matthew-Wood syndrome (MWS) / Microphthalmia, Syndromic 9 (MCOPS9) (OMIM #601186) due to overlap of some phenotypic features may suggest that some male foetuses without a genetic diagnosis of MWS due to *STRA6* mutation, may have mutation of *PORCN*.

PORCN (Porcupine) is involved in the trafficking of Wnt proteins between the ER and Golgi, as shown in Figure 8, adapted from reviewed in (Clements, 2009). Defective PORCN impairs transfer of Wnt proteins through the cell leading to reduced Wnt secretion affecting

downstream pathways reliant on Wnt signalling. The mutation observed likely encodes a hypomorphic allele with reduced function. Wnt signalling is essential for many aspects of embryonic development and this explains the constellation of congenital anomalies in multiple organs. A conditional mouse model of PORCN was used to study its requirement in Wnt signaling and embryonic development (Barrott et al., 2011). Consistent with the female-specific inheritance pattern of FDH, Porcn hemizygous male embryos arrest during early embryogenesis and fail to generate mesoderm, a phenotype previously associated with loss of Wnt activity. Heterozygous Porcn mutant females exhibit a spectrum of limb, skin, and body patterning abnormalities resembling those observed in human patients with FDH. In a conditional mouse model of PORCN loss of function defects were observed in ectodermal and mesenchymal derived structures (Liu et al., 2012). It has been proposed that CDH originates from a defect in the mesenchymal cells of the developing pleuroperitoneal folds (PPFs) and this provides a plausible mechanism for the development of CDH seen in one of our patients.

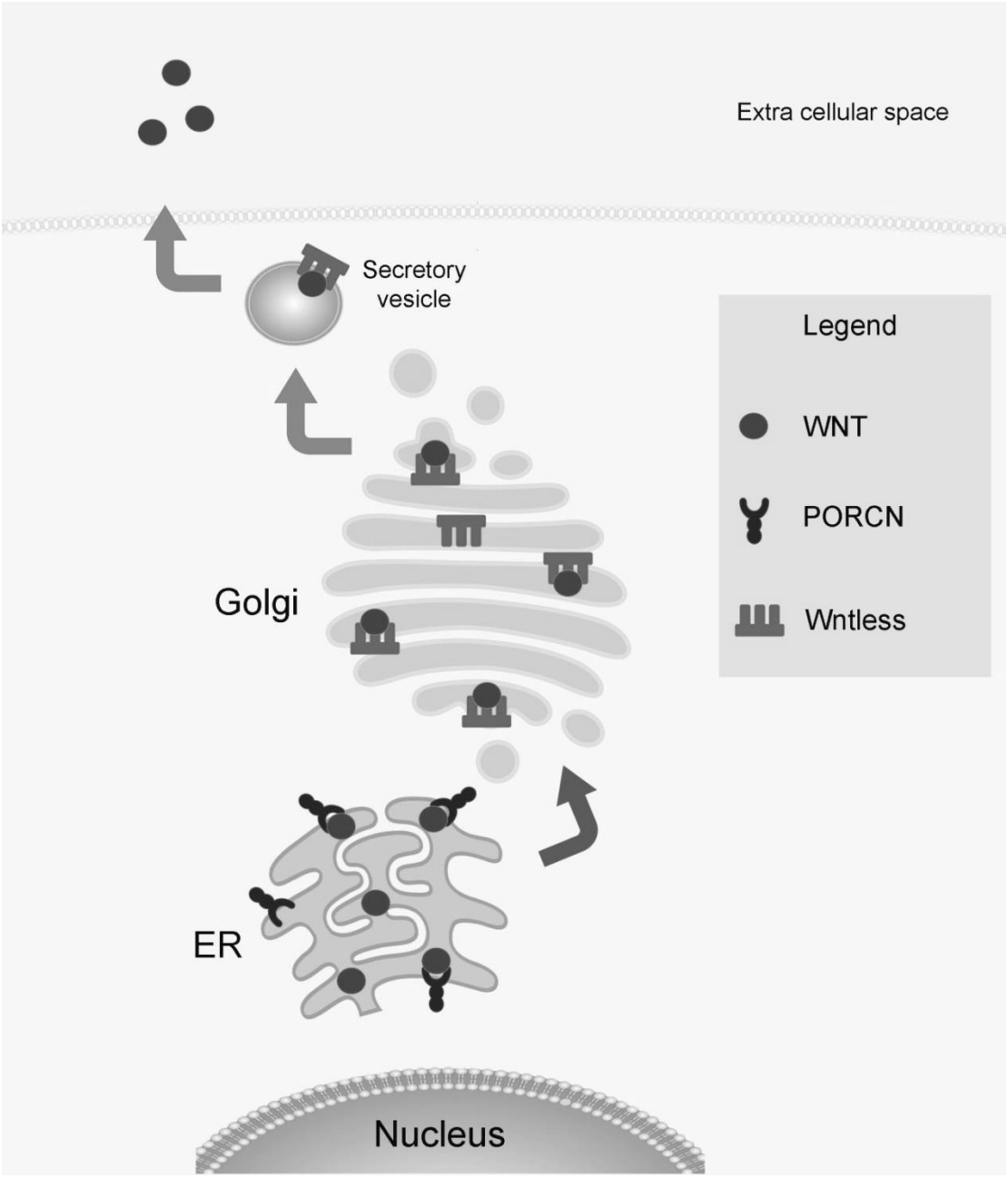


Figure 8. Provides an overview of the role of PORCN in the trafficking of WNT proteins through the ER to the golgi. WNT proteins are glycosylated and lipid modified by PORCN (Porcupine) in the ER, where they are subsequently escorted through the Golgi to the plasma membrane for secretion by Wntless. Defective PORCN results in reduced WNT secretion.

Results: Family 3

Chromosomal microarray analysis did not reveal any pathogenic CNVs in the fetus referred due to MCA including CDH. Due to the consanguineous relationship of the parents a genetic cause was suspected and we undertook exome sequencing in the parent-fetus trio (I.1, I.2, and II.1). We assumed a homozygous recessive mutation as the likely cause given the consanguineous relationship.

Variant filtering was performed with two approaches; using excel, and using annotate-it (Sifrim et al., 2012). Annotated variant files for the affected individual (II.2) were filtered in Excel on quality parameters, variant type, and by dbSNP137 and/or the ESP6500 annotation. Variant frequency was also used to identify homozygous mutations. Of 3 splice variants remaining after variant filtering, a homozygous splice variant was identified in the affected patient as a plausible candidate; PIGN (NM_012327:exon17:c.1574+1G>A, NM_176787:exon18:c.1574+1G>A). Checking the variant files of both parents revealed that they both carry the heterozygous form. Details of variant filtering are given in table 12.

Annotate-it was also used to identify candidate variants assuming a homozygous recessive or *de novo* cause. The variant filtering strategy using annotate-it is provided in Table 13. No likely candidate variants were identified assuming a de novo mutation as the possible cause. Searching for a homozygous recessive mutation as the cause revealed a candidate splice variant in the *PIGN* gene present in homozygous form in the affected foetus (II.1) and present in heterozygous state in both parents (I.1 and I.2). The splice mutation identified as a possible candidate has been observed once in the exome variant server database (var.freq. 0.000083) in heterozygous form, but is seen in homozygous state in our patient. Details of the candidate variant are shown in Table 14, and the pedigree in Figure 9.

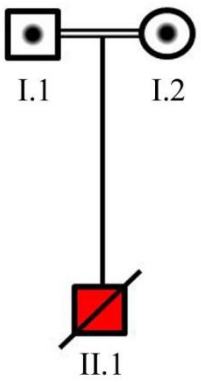


Figure 9. Family 3 Pedigree; All individuals I.1, II.2, and II.1 underwent exome-sequencing which revealed the c.1574+1G>A splice mutation in the *PIGN* gene in all individuals, present in homozygous form in the affected foetus (II.1) and heterozygous form in the parents (I.1 and I.2).

			II.1
		Total number of Variants	**81698**
	Quality Filter	Depth > 10 Q (phred) > 30	46200
Variant Type	Non-synonymous	total Not dbSNP137 Rare Variant <1% ESP6500 dbSNP137 & ESP6500 var freq. >0.8 SIFT PolyPhen Mutation Taster Predictions combined	11829 259 431 30 3
	Stop Gain	Total Not dbSNP137	171
	Stop Loss	Total Not dbSNP137	66
	Exonic Splicing	Total Not dbSNP137 Rare Variant <1% ESP6500 var freq. >0.8	7144 671 26 **3**
	Frameshift substitution	total	114

Table 12. Excel Variant Filtering

		Consanguineous Homozygous Recessive Variant	**De novo**
Included Samples		II.1, I.1, I.2 In at least 3 samples	II.1
Excluded Samples		None	I.1, I.2
Filter Included Samples		Min. Depth: 10 Var. Freq.: 30-100%	Min. Depth: 10 Var. Freq.: 30-100%
Filter Excluded Samples		None	Min. Depth: 10 Var. Freq.: 30-100%
Variant Type	Nonsense Splice Site Non-synonymous Synonymous UTR	Yes Yes Yes No No	Yes Yes Yes No No
dbSNP		Not used	Not used
1000genomes	Population Frequency	< 1%	< 1%
200 Danish Exomes		Not used	Not used
Prediction Criteria	SIFT Polyphen2 LRT Mutation Taster	NO [YES]	NO [YES]
Total number of Variants		**48** [0]	32 [0]

Table 13. Annotate-it Variant Filtering

	Affected	Father	Mother
Chromosome	18	18	18
Position	59777066	59777066	59777066
Gene Name	PIGN	PIGN	PIGN
Depth	15	16	15
Reference Allele	C	C	C
Number of reads with Ref. allele [freq.]	0 [0]	6 [0.37]	10 [0.66]
Alternate Allele	T	T	T
Number of reads with Alt. allele [freq.]	15 [1.0]	10 [0.62]	5 [0.33]
Mutation Type	Splicing	Splicing	Splicing
Refseq accession ID	NM_012327:exon17; NM_176787:exon18	NM_012327:exon17; NM_176787:exon18	NM_012327:exon17; NM_176787:exon18
Mutation DNA	c.1574+1G>A	c.1574+1G>A	c.1574+1G>A
Mutation RNA	NM_012327:exon17 Exon skipped	NM_012327:exon17 Exon skipped	NM_012327:exon17 Exon skipped
Mutation Protein	Predicted frameshift and truncated protein	Predicted frameshift and truncated protein	Predicted frameshift and truncated protein
Functional Prediction - SIFT - Polyphen2 - LRT - Mutation Taster	None	None	None
Confirmed by Sanger Seq	Yes	Yes	Yes
EVS: Number [freq.]	Homozygous form not observed	1/12029 [0.000083]	1/12029 [0.000083]

Table 14. Variant Details for Main Candidate

In order to determine the consequences of the PIGN splice site mutation exon junction primers were designed spanning exons 15/16 and exons 18/19 (NM_012327). cDNA was reverse transcribed from mRNA isolated from cultured foetal skin fibroblasts from the affected patient. Exon junction PCR on the cDNA sample demonstrated a PCR product of ~310bp in size consistent with skipping of exon 17 as shown in Figure 10.

Exon-Junction PCR

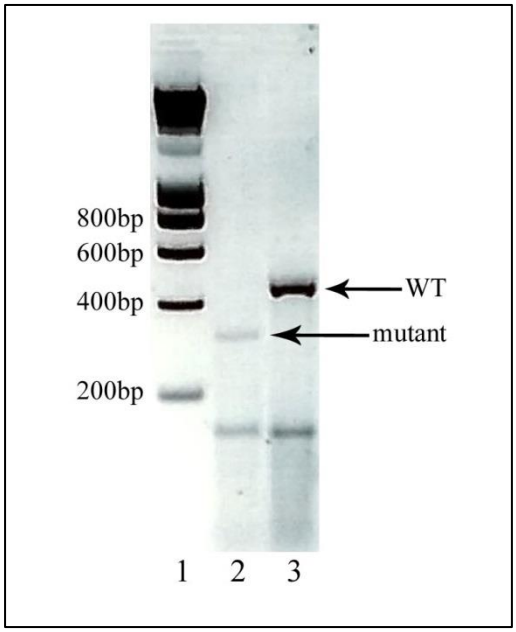


Figure 10. Agarose gel image of the PCR products obtained from exon junction RT-PCR in the patient and a control sample. Lane 1 = Ladder; Lane 2 = Patient sample; Lane 3 = Control sample. The Control sample shows a PCR product of ~450bp, consistent with the expected product size. The patient sample shows a PCR product of ~310bp, consistent with exon skipping of exon 17.

In order to confirm this interpretation and to check for the potential involvement of a cryptic splice site, this PCR product was sequenced by conventional Sanger sequencing which confirmed this interpretation of skipping of exon 17. This novel splicing event is predicted to cause a frameshift due to the aberrant splicing of exons 16 and 18 which would be predicted to lead to premature truncation of the protein, as shown in Figure 11.

Predicted effect on Protein

←========== Exon 16 ==========→ ←==========
VSLCKELIHLALKGLSYYHTYDRFFLGVNVVIGFVGWISYASLLIIKSHSNLIKGVSKEVK KPSHLLPCSFVAIGILVAFFL
VSLCKELIHLALKGLSYYHTYDRFFLGVNVVIGFVGWISYASLLIIKSHSNLIKGVSKEVK ----------

=Exon 17 ==========→ ←==========Exon 18==========→
LIQACPWTYYVYGLLPLPIWYAVLR EFQVIQDLVVSVLTYPLSHFVGYLLAFTLGIEVL
---------- **ISSYSGPCCISVDLSSEPFCWVPVSLYPGNX**

Figure 11. Displays part of the amino acid sequence for the PIGN surrounding the splice site mutation. The location of exon 16, 17 and 18 is shown on the top row; the normal amino acid sequence is displayed on the middle row; and the predicted amino acid sequence generated by the splice site mutation on the bottom row. The mutation is predicted to cause a frameshift due to exon skipping of exon 17, and the splicing of exons 16 and 18, leading to a premature stop codon after 30 novel amino acids translated from the splicing of exon 16 and 18.

Discussion: Family 3

Using exome sequencing we identify a homozygous splice mutation in the *PIGN* gene in a foetus from consanguineous parents, affected with multiple malformations including bilateral diaphragmatic hernia. Recently nonsynonymous PIGN mutations in humans were identified as a cause of multiple congenital anomalies-hypotonia-seizures syndrome [OMIM #614080] in a large consanguineous family with multiple affected individuals (Maydan et al., 2011). CDH was not described in any of the affected individuals. Our report of a second consanguineous family with a mutation in *PIGN* thus adds to the phenotypic spectrum observed with pathogenic autosomal recessive PIGN variants. This suggests that complete loss-of-function nonsense, or splicing mutations, in PIGN can cause severe multiple congenital anomalies, including CDH, which are likely to be lethal in the embryonic or neonatal phase. The present case shows phenotypic overlap with the foetuses with a suspected diagnosis of Fryns syndrome reported by Ramsing et al (Ramsing et al., 2000), including; diaphragmatic hernia, oligodactyly, and cardiac defects. This raises the possibility that some cases with MCA including CDH, which may be clinically classified as Fryns syndrome (or-Fryns-like), may be due to autosomal recessive mutations in *PIGN*, or other genes involved in the GPI-anchor biosynthesis pathway. In our case the homozygous splice site mutation predicting a truncated protein is likely responsible for the increased severity of the phenotypic features in comparison to the previous report of nonsynonymous mutations which may produce hypomorphic alleles. However, further reports are necessary to determine the full phenotypic spectrum associated with different types of variants in *PIGN*.

PIGN is involved in GPI anchor biosynthesis. Glycosylphosphatidylinositol (GPI) anchors allow the attached protein to anchor to the outer leaflet of the cell membrane, and perform a diverse set of functions including roles in signal transduction, cell adhesion and antigen presentation. GPI anchors are assembled stepwise on phosphatidylinositol (PI) in the ER membrane, as shown in Figure 12. PIGN encodes one of the ethanolamine phosphate (EtNP) transferases and has been demonstrated to be essential for the addition of the first EtNP unit to the first mannose unit (Hong et al., 2005; Hong et al., 1999; Yada et al., 2001). A number of the genes involved in GPI anchor synthesis have recently been identified as causing phenotypes in humans, many by exome sequencing analysis, including; PIGA (OMIM*311770), PIGL (OMIM*605947), PIGM (OMIM*610273), PIGN (OMIM*606097), PIGO (OMIM*614730), PIGT (OMIM*610272), and PIGV (OMIM*610274), summarized in Table 15. Mutations in many of these genes share overlapping features of MR/ID, seizures, cardiac defects, skeletal defects, and dysmorphic features. The defective GPI anchor caused

by mutations in these genes results in the subsequent mis-localisation of GPI anchored proteins. While there is some degree of overlap between these different syndromes, there is clearly phenotypic variability between those features depending on which step in the GPI anchor synthesis pathway is affected.

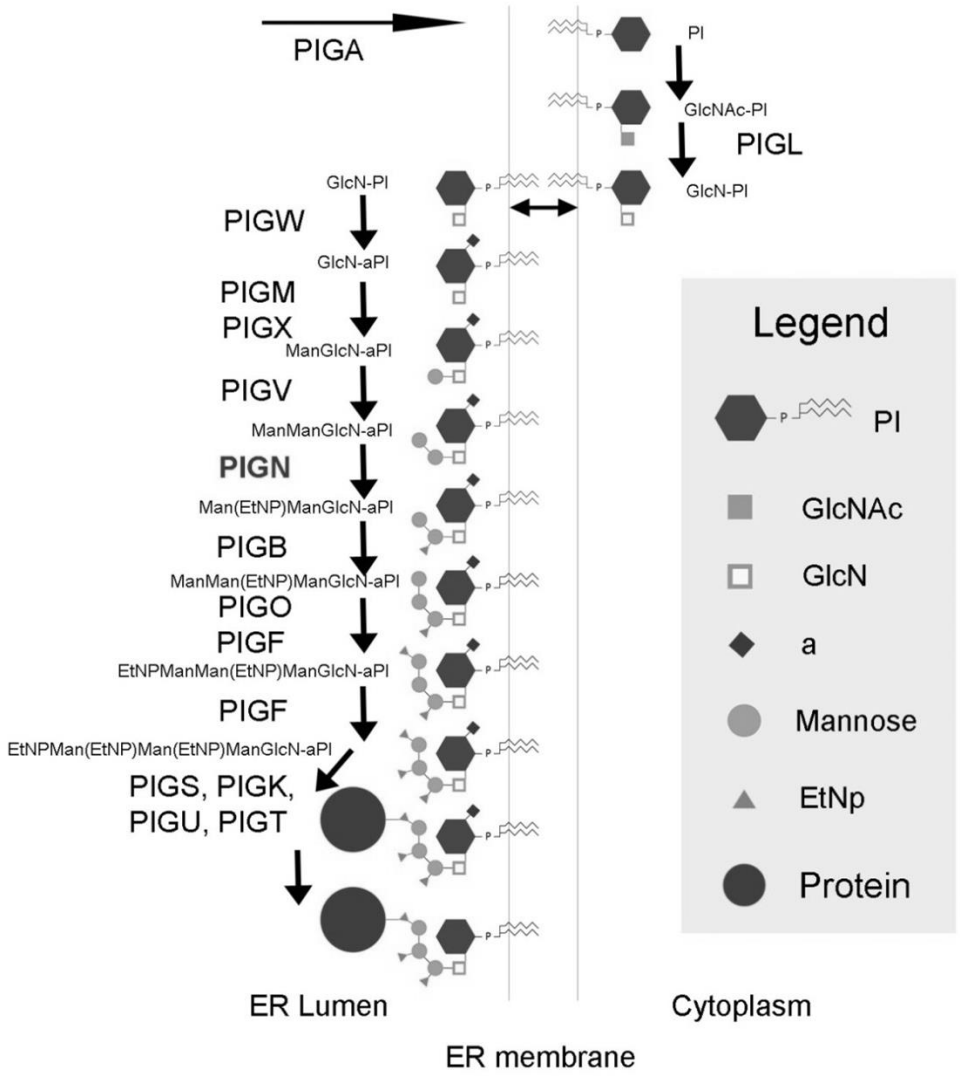


Figure 12. Shows a schematic representation of GPI anchor biosynthesis. PIGA transfers phosphatidylinositol (PI) to the cytoplasmic face of the ER, where PIGL undertakes de-N-acetylation of N-acetylglucosaminylphosphatidylinositol (GlcNAc-PI). GlcN-PI is flipped to the ER lumen, where PIGW adds an acyl chain (a) to inositol (GlcN-aPI). PIGM is a mannosyltransferase that adds the first mannose (Man) to the core GPI, followed by addition of a second mannose by PIGV. PIGN (highlighted) encodes an ethanolamine phosphate transferase, which adds the ethanolamine phosphate (EtNP) to the first mannose on the GPI anchor. PIGB adds a third mannose, to which PIGO adds an EtNP, and PIGF adds EtNP to the second mannose. Several PIG genes are involved in anchoring of the entire sugar-lipid unit to proteins with the appropriate C-terminal amino acid sequence using a multi-subunit transamidase complex. A de-acylase finally removes the acyl chain generated by PIG-W.

Gene	Function	Phenotypic Features	OMIM syndrome	Method	Reference
PIGA	GlcNAc-PI synthesis	Cleft palate, seizures, contractures, brain structural malformations, early death	#300868 Multiple congenital anomalies-hypotonia-seizures syndrome 2	Exome	(Johnston et al., 2012)
PIGL	GlcNAc-PI de-N-acetylase	Coloboma, congenital heart disease, ichthyosiform dermatosis, intellectual disability, hearing loss	#280000 CHIME syndrome	Exome	(Ng et al., 2012)
PIGM	Mannosyl transferase	venous thrombosis and seizures	#610293 Glycosylphosphatidyl-inositol deficiency	Mapping	(Almeida et al., 2006)
PIGN	GPI ethanolamine phosphate transferase	Hypotonia, psychomotor delay, seizures, dysmorphic features, anomalies in cardiac, urinary, and gastrointestinal systems.	#614080 Multiple congenital anomalies-hypotonia-seizures syndrome 1	Mapping	(Maydan et al., 2011)
PIGO	GPI ethanolamine phosphate transferase	moderate to severe psychomotor developmental delay, facial dysmorphism, brachytelephalangy, hyperphosphatasia. Additional features include cardiac septal defects and seizures	#614749 Hyperphosphatasia with mental retardation syndrome 2	Exome	(Krawitz et al., 2012)
PIGT	Catalysis of protein attachment	distinct facial features, intellectual disability, hypotonia and seizures, in combination with abnormal skeletal, endocrine, and ophthalmologic findings	No OMIM entry A novel autosomal recessive intellectual disability syndrome	Exome	(Kvarnung et al., 2013)
PIGV	Mannosyl transferase	mental retardation, various neurologic abnormalities such as seizures and hypotonia, and hyperphosphatemia. Additional features include facial dysmorphism and brachytelephalangy	#239300 Hyperphosphatasia with mental retardation syndrome 1	Exome	(Krawitz et al., 2010; Horn et al., 2011)

Table 15. Lists the genes involved in GPI anchor synthesis in which pathogenic mutations have been found in humans, along with the associated phenotypic features, and reference to the OMIM listed syndrome. Adapted and updated from (Freeze, 2013).

A recent study investigated the effect of two mouse models, the Pign (gonzo mutant) and the Pgap1 (beaker mutant), on Cripto signalling in forebrain development as a model of holoprosencephaly (McKean and Niswander, 2012). Cripto is a GPI anchored protein, and is an obligate Nodal co-factor involved in TGFβ signalling. Defects in Cripto (mouse) and the human ortholog TDGF1 have previously been associated with forebrain defects, and Cripto is thus considered a key GPI anchored protein which causes an HPE-like phenotype (McKean and Niswander, 2012). Interestingly the authors demonstrate that TGFβ signalling is reduced both in vitro and in vivo in these models. TGF-β1 null mice develop apparently normally, but soon die from aggressive pulmonary or gut inflammation due to a failure to negatively modulate the immune system (McLennan et al., 2000). Disruption of TGF-β signaling in TAB1 null mice results in lethal cardiovascular and lung abnormalities (Komatsu et al., 2002). Abnormal diaphragm morphology has also been reported in a Tgfb1 mouse model; the diaphragm showed inflammation and necrosis which were severe enough to interfere with normal respiration (Shull et al., 1992).

Conclusion

We demonstrate that exome sequencing can identify pathogenic variants associated with both syndromic and isolated CDH where a familial origin is suspected due to multiple affected individuals or consanguinity. In cases of isolated-CDH, with no prior family history, the recurrence risk for future pregnancies is often quoted as 1-2%. However, the application of exome sequencing (following a normal chromosomal microarray analysis) may identify deleterious mutations which thus allow for a more accurate prediction of recurrence risk, which may be up to 50% for CDH or other congenital anomalies in some families.

We add further evidence for association of *ZFPM2* with CDH by identification of a heterozygous nonsense mutation in *ZFPM2* as a cause of familial isolated CDH in 2 affected siblings. This mutation displays variable phenotypic expression being present in a third sibling with a mild diaphragmatic eventration and a TAPVR. The same variant is seen in the mother and 2 additional family members, all of whom are asymptomatic, thus highlighting that *ZFPM2* haploinsufficiency is associated with reduced penetrance. Our finding adds further evidence for *ZFPM2* having a role in diaphragm and cardiovascular development, defects in which are associated with an autosomal dominant effect, but with reduced penetrance and variable expression. We add evidence for *PORCN* mutations in males as a cause of multiple congenital anomalies including microphthalmia and CDH by the identification of a non-synonymous mutation in *PORCN* as a cause of Focal Dermal

Hypoplasia (FDH) in 2 male foetuses. This represents the first report of *PORCN* mutations in affected non-mosaic males, highlighting a spectrum of phenotypic features including microphthalmia, CDH, neural tube defects, and cardiac anomalies. The mutation is inherited from the mother who showed extreme skewing of X-inactivation. Two female siblings carried the same mutation, one of whom displays mild features of FDH. We also identified a homozygous splice site mutation in *PIGN* in a foetus with multiple congenital anomalies including bilateral CDH. Our finding expands the phenotypic spectrum associated with homozygous loss of function mutations in *PIGN*, and adds further support for defective GPI anchor biosynthesis as a cause of developmental abnormalities.

Our findings demonstrate that exome sequencing is a valuable approach for the identification of a genetic cause for isolated and non-isolated CDH due to inherited mutations. However, conventional methods of variant filtering remain challenging for disorders which display reduced penetrance and variable expression between individuals, particularly in small family pedigrees with perhaps only a single affected individual.

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Chapter 6

Gene Expression Analysis of CDH Patients

Chapter 6. Gene Expression Analysis of CDH Patients

Adapted from;

Gene Expression Profiling by RNA-Sequencing of Amniotic Fluid Cells Distinguishes Different Classes of Isolated CDH Foetuses. (Manuscript in preparation)

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Introduction

Congenital diaphragmatic hernia (CDH) occurs with an incidence of 1.7 to 5.7 per 10,000 live-born infants (Kotecha et al., 2012), and is associated with pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality and much of the morbidity in survivors. CDH is observed in association with additional anomalies in ~40% of cases, i.e. syndromic or non-isolated CDH, for which the prognosis is worse (Skari et al., 2000). For the remainder, CDH occurs as an isolated defect and it is those patients who are most suited to prenatal or neonatal therapy.

Currently, a number of clinical observations and biometrics in both the prenatal and neonatal phase are used as biomarkers of *isolated* CDH severity and certain predictors of outcome. Predictions based on the lung area to head circumference ratio, and the liver position, are among the best studied. Jani et al showed that significant predictors of survival were the observed to expected lung to head ratio (O/E LHR), gestational age at delivery, and the side of CDH (i.e. left or right; with ~85% occurring on the left) (Jani et al., 2007). Jani et al later investigated the value of the O/E LHR and liver position as predictors of neonatal morbidity in *survivors* with isolated CDH (Jani et al., 2009). The O/E LHR provided significant prediction of the need for prosthetic patch repair, duration of assisted ventilation, need for supplemental oxygen at 28 days, and incidence of feeding problems. An additional independent prenatal predictor of the need for patch repair was herniation of the liver into the thorax.

This information is highly relevant in the counselling of patients, assisting with decision for either; prenatal intervention by fetoscopic endoluminal tracheal occlusion (FETO) which aims to reverse pulmonary hypoplasia for the most severe cases; expectant management and neonatal therapy; or the option for termination of pregnancy (Deprest et al., 2009). Deprest et al proposed four categories of classification for isolated left-side CDH based upon the observed to expected lung to head ratio (O/E LHR), and also taking into account liver position (extreme; severe; moderate; and mild) (Deprest et al., 2009); (i) foetuses with an O/E LHR <15% have *extreme pulmonary hypoplasia* and (virtually) no survivors are reported; (ii) foetuses with an O/E LHR between 15 and 25% have *severe pulmonary hypoplasia* and their predicted survival is ~20%, those with the liver down doing better than those with liver into the thorax; (iii) foetuses with an O/E LHR between 26% and 35%, and those with an O/E LHR between 36 and 45% but with the liver up, have *moderate pulmonary hypoplasia* and have an expected survival rate between 30% and 60%, depending on the size of the lung; and (iv) foetuses with an O/E LHR 36 and 45% with the liver down, and those with an O/E LHR >45%, have *mild pulmonary hypoplasia* and they are very likely to survive (>75%).

Recently, Done et al investigated neonatal morbidity in fetuses with severe isolated CDH treated with FETO versus those managed expectantly (Done et al., 2013). Early neonatal morbidity indicators including the need for patch repair, duration of mechanical ventilation and supplemental oxygen, age at full enteral feeding and incidence of pulmonary hypertension were investigated. Gestational age at delivery was predictive of the duration of assisted ventilation, number of days on supplemental oxygen, and age at full enteral feeding. Delivery after 34 weeks gestation was correlated with neonatal morbidity of FETO cases comparable to that of expectantly managed cases with moderate hypoplasia (Done et al., 2013).

Studies of chromosomal abnormalities and the identification of single gene disorders in the group of non-isolated or syndromic CDH patients shows there is a strong genetic component, with as much as 20-30% having an identifiable genetic cause (Howe et al., 1996; Enns et al., 1998; Witters et al., 2001; Holder et al., 2007; Scott et al., 2007; Wat et al., 2011; Yu et al., 2012). However, large chromosomal abnormalities are not frequently seen in association with isolated CDH, and causative genes for isolated cases remain elusive. We have recently shown that chromosomal microarray analysis identified pathogenic submicroscopic copy number variations (CNVs) in ~10% of foetuses with moderate-severe isolated CDH (Brady et al., 2013), and we also identified *ZFPM2* as a cause of familial isolated

CDH (Brady et al., manuscript submitted). However, for the vast majority of isolated CDH patients a genetic cause remains unidentified by chromosomal microarray analysis and exome sequencing has not yet been applied to cohorts of sporadic cases of isolated CDH.

Clinically, it would be useful to timely identify good responders and poor responders to the currently available foetal therapy, and the genetic factors influencing this response. The identification of genetic biomarkers from amniotic fluid (AF), or even from maternal blood or plasma, offers the possibility of a more personalised foetal therapy. We sought to explore whether gene expression analysis in AF cells of CDH foetuses using RNA-Sequencing could identify dysregulated genes and biological pathways which could be correlated with, and are thus predictive biomarkers, of factors such as severity, response to FETO therapy, or neonatal outcome.

Methods

Patients

Retrospective samples were obtained from 23 male foetuses with moderate to severe isolated CDH, and with normal karyotype and/or chromosomal microarray. These included 14 fetuses with left-sided CDH, and 9 fetuses with right-sided CDH. In order to minimize the effects of different gestational ages, samples were selected in the range of 25-29 weeks, and prior to FETO (where performed). Cultured amniotic fluid (AF) cells were obtained from frozen cell cultures. As a control, AF samples of normal foetuses from twin-twin transfusion pregnancies from the same gestational age range were selected (n=8).

RNA Isolation

Total RNA was isolated from cultured AF cells with the RNeasy mini kit (Qiagen) according to the manufacturer's recommendation. Briefly, samples were washed twice in PBS and the final cell pellet was resuspended in 600µl Buffer RLT, using a syringe for lysis and homogenization. The same volume of 70% EthOH was added to the lysate which was passed through a single spin column. Following 3 wash steps, total RNA was eluted in 55µl RNase free water. RNA concentration was measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific). RNA integrity was assessed using the RNA 6000 Nano Kit and the Bioanalyser (Agilent Technologies) according to the manufacturer's recommendations.

RNA-Seq Library Preparation & Sequencing

mRNA isolation, cDNA conversion and sequencing library preparation was performed using the TruSeq RNA library preparation kit (Illumina) according to the manufacturer's recommendations for the low throughput manual protocol, with the gel-free method, and the following modifications; fragmentation time was reduced from 8 minutes to 6 minutes; the enrichment PCR step was reduced from 15 to 12 cycles. 2µg total RNA was used as initial input material.

Library concentrations were measured using the Qubit fluorometer (Life Technologies). Library quality and size range was assessed using the Bioanalyser (Agilent Technologies) with the DNA 1000 Kit (Agilent Technologies) according to the manufacturer's recommendations. Conversion to molar concentrations was calculated suing the cynosura calculator; (http://www.cynosura.org/molbiol/scripts/01_07.html), with final concentrations of 2nM provided for sequencing on the HiSeq 2000 (Illumina). Samples were indexed during library preparation and subsequently pooled 12 per lane of a sequencing flow cell.

Bioinformatics Analysis

After demultiplexing and quality filtering, fastq files were imported into Array Studio (OmicSoft) and mapped against the ensembl GRCh37 reference human genome and transcriptome (ensembl release 66) using default parameters for single short read data. Expression values were calculated per gene; normalized to 'reads per kilobase per million reads' (RPKM) values as previously described (Mortazavi et al., 2008). This measurement has been criticised, but is widely used for analysis of RNA-Seq data (Wagner et al., 2013; Wagner et al., 2012). An advantage of RNA-Seq analysis compared to expression arrays is there is no background signal noise, so a low number of sequencing reads theoretically equates to gene expression. However, it has been proposed that RNA-Seq identifies one group comprised of very low abundance and putatively non-functional mRNAs, and another group of highly expressed mRNAs with active chromatin marks at their promoters (Hebenstreit et al., 2011). In order to address this, we apply a cutoff of 1RPKM to distinguish between 'noisy' or 'leaky' transcription and 'true' mRNA expression levels for comparison between samples.

In order to proceed with conventional expression analysis methodologies developed for expression array data we Log2 transform the RPKM values for downstream clustering

analysis by principal component analysis (PCA) and by hierarchical clustering, and for the subsequent calculation of fold changes by statistical inference analysis.

Pathway Analysis

Pathway analysis was performed using the Ingenuity Pathway Analysis (IPA) software application (Ingenuity Systems, CA, USA).

Generation of Networks and Calculation of p-values and Scores using the IPA Software.

(i) Network Algorithm and Network Scores;

The IPA software applies an algorithm for generating networks from uploaded gene sets (gene lists, or with associated expression values) by mapping each gene identifier to its corresponding gene object in the IPA Knowledge Base (comprising data from multiple curated evidence sources). These focus genes are then overlaid onto a global molecular network developed from information contained in the Knowledge Base. Networks of these focus genes are then algorithmically generated based on their connectivity. Each individual IPA network has a maximum of 35 focus genes and is assigned a significance score (based on the p-value) representing the likelihood that the focus genes within the network are enriched for by random chance alone. A high number of focus genes within a dataset leads to a higher network score, where the score is equal to –log(p-value), such that a score of 3 or higher corresponds to a p-value of 10E-3 and higher.

(ii) Calculating and Interpreting the p-values for Biological Function and Canonical Pathways in IPA;

The p-value associated with a function or a pathway in Global Functional Analysis (GFA) and Global Canonical Pathways (GCP) is a measure of the likelihood that the association between a set of focus genes in and a given process or pathway is due to random chance alone. In general, p-values less than 0.05 indicate a statistically significant, non-random association. The p-value is calculated using the right-tailed Fisher Exact Test.

In this method, the p-value for a given process annotation is calculated by considering (i) the number of focus genes that participate in that process, and (ii) the total number of genes that are known to be associated with that process in the selected reference set (in this case all information in the IPA Knowledge Base). The more focus genes which are involved, the more likely the association is not due to random chance alone and the more significant the p-value. In this way, the larger the total number of genes known to be associated with the

process, the greater the likelihood that an association is due to random chance, and the p-value accordingly becomes less significant. The p-value thus identifies statistically significant over-representation of focus genes in a given process. Over-represented functional or pathway processes are processes which have more focus genes than expected by chance using the right-tailed Fisher Exact Test.

(iii) Downstream Effects Analysis, Upstream Regulator Analysis and Mechanistic Network Analysis;

The IPA Downstream Effects Analysis predicts increases or decreases in downstream biological activities occurring in the tissues or cells under study. Using the directional expression changes of the genes in the respective dataset, IPA computes the likely effect (increase of decrease) on diseases and cell biological processes. The IPA Upstream Regulator Analysis predicts upstream regulators using the directional expression changes of the underlying data. Mechanistic Networks Analysis takes this analysis further by computationally generating plausible directional networks from these regulators.

Results

PCA, Hierarchical Clustering Analysis and Identification of Sub-Classes for Fold Change Calculation

At least 10 million reads were obtained for all samples (range 12-28 million reads), with 89-92% of reads mapping uniquely to the reference human genome / transcriptome. Normalised expression values per gene (RPKM values) were filtered; genes with <1 RPKM in all samples were excluded from further analysis (for reasoning, see methods), leaving 10,792 protein coding genes. The RPKM values for these 10,792 genes were subsequently log2 transformed. Correlation analysis identified one control sample to be a clear outlier, which was removed from subsequent analysis. Unsupervised hierarchical clustering and PCA on the remaining 7 control samples and 23 CDH samples was performed. This could not clearly identify any clustering of, controls vs CDH, nor left vs right CDH. No batch effect could be identified due to factors including cell culture batches, RNA isolation batches, library preparation batch, or total number of reads obtained.

Statistical inference analysis was performed using the general linear model function in Array Studio to generate fold changes and FDR values (false discovery rate; BH; Benjamin-Hochberg corrected p-values). Group analysis comparing all 23 CDH patients vs 7 controls revealed no significant fold changes with cutoff of fold change +-2, and FDR <0.01. Similarly,

analysis comparing 14 left-sided CDH vs controls, and 9 right-sided CDH vs controls revealed no significant fold changes using the same cutoffs.

In order to further stratify the CDH group into potential subtypes with similar molecular profiles, comparison was made to the control group per individual CDH patient (assuming equal variance of expression as for the control group for the respective gene). By applying a cutoff p-value of <0.1, >4000 genes showed a fold change of >2, or <-2. PCA and unsupervised hierarchical clustering were performed on this subset of 4226 genes which show an apparent fold change in any CDH sample vs the control group. This allowed for the identification of several homogenous subgroups, 2 of which contained at least 5 CDH cases, and one group which contained only 2 cases and was not further analysed. An additional 2 control samples were identified as outliers from the remaining control samples, and were removed from subsequent analysis, leaving 5 control samples with more homogeneous expression profiles. Inference analysis was then repeated on the 10,792 genes from above, but comparing the 2 defined CDH groups against the newly defined control group. Those groups herein referred to as group2 and group7, showed significantly dysregulated gene expression, and are shown in the PCA plot in Figure 6.1. Group2 contains 6 isolated CDH cases; 2 with left-sided CDH and 4 with right-sided CDH, 5 of which survived and 1 foetus deceased neonatally (right-sided CDH). Group7 contains 5 isolated CDH cases; 4 with left-sided CDH and 1 with right-sided CDH, 4 of which survived and 1 deceased neonatally (right-sided CDH).

Only 32 genes were dysregulated with a fold change +-2, and FDR 0.01, in group2. Using the same fold change +-2, but FDR of 0.05, 101 genes were dysregulated. This list of 101 genes was used for the subsequent downstream pathway analysis. In contrast, in group7, 250 genes were found to be dysregulated with fold change +-2, and FDR 0.01. Using the same fold change +-2, but FDR of 0.05, 374 genes were dysregulated. We used this list of 374 genes for the downstream pathway analysis. These 2 groups share in common 35 dysregulated genes; however the direction of expression change between these two groups is not the same for many of these genes. The details of the top 10 upregulated and top 10 downregulated genes are listed in Tables 6.1, and Table 6.2, below. The details for genes in common between group2 and group7 are given in Table 6.3. Figure 6.2 displays a heatmap generated by unsupervised hierarchical clustering of the 440 total genes which were significantly differentially expressed in group 7 and/or group 2 in comparison to the control group.

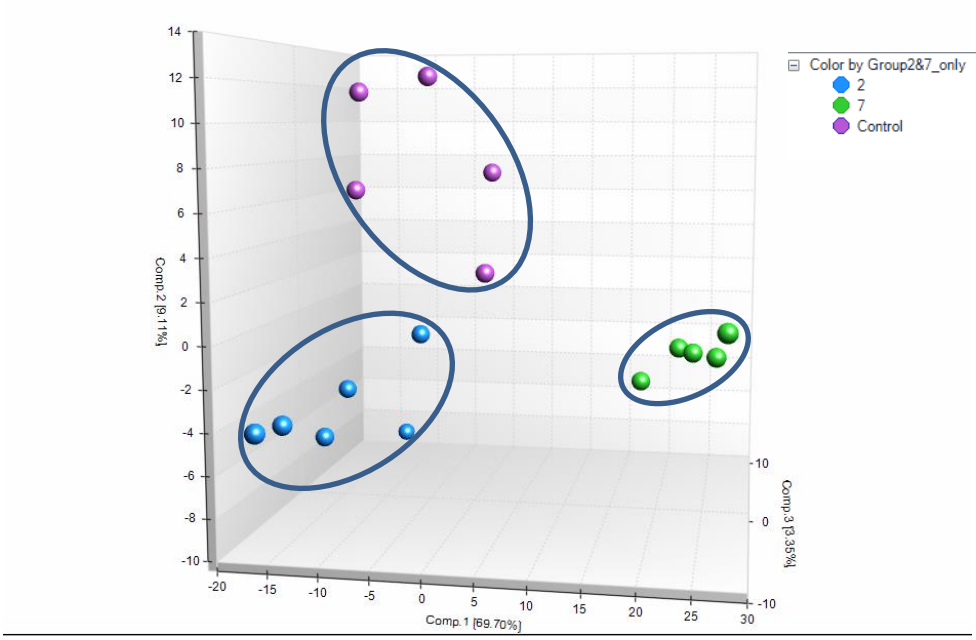


Figure 6.1. PCA for subgroup 2 (6 isolated CDH), subgroup 7 (5 isolated CDH), and 5 control samples. Subgroup 7 separates from the control group (as well as subgroup 2) on the first component (Comp1; x-axis), and subgroup 2 separates from the control group on the second component (Comp2; y-axis).

	Ensembl Gene ID	Gene Name	Chr	Start	End	Fold Change	FDR (BH)
Downregulated	ENSG00000108753	HNF1B	17	36,046,434	36,105,237	-6.6739	0.001
	ENSG00000171234	UGT2B7	4	69,917,081	69,978,705	-6.2276	0.0027
	ENSG00000132182	NUP210	3	13,357,737	13,461,809	-4.9789	0.001
	ENSG00000106003	LFNG	7	2,552,163	2,568,811	-4.5124	0.0039
	ENSG00000126878	AIF1L	9	133,971,863	133,998,539	-4.3895	0.0029
	ENSG00000075891	PAX2	10	102,495,360	102,589,698	-4.3415	0.0016
	ENSG00000085563	ABCB1	7	87,132,948	87,342,611	-4.1651	0.0106
	ENSG00000146038	DCDC2	6	24,171,984	24,358,280	-3.8992	0.0055
	ENSG00000197635	DPP4	2	162,848,751	162,931,052	-3.8738	0.0241
	ENSG00000156510	HKDC1	10	70,980,059	71,027,315	-3.7977	0.0029
Upregulated	ENSG00000112769	LAMA4	6	112,429,963	112,576,141	2.7279	0.026
	ENSG00000170801	HTRA3	4	8,271,492	8,308,838	2.7359	0.0237
	ENSG00000113083	LOX	5	121,398,890	121,414,206	2.7761	0.0116
	ENSG00000049323	LTBP1	2	33,172,039	33,624,576	2.786	0.0404
	ENSG00000143867	OSR1	2	19,551,246	19,558,414	2.8778	0.0188
	ENSG00000185112	FAM43A	3	194,406,622	194,409,762	2.9923	0.0119
	ENSG00000147027	TMEM47	X	34,645,181	34,675,405	3.1498	0.0132
	ENSG00000157404	KIT	4	55,524,085	55,606,881	3.6289	0.0088
	ENSG00000108821	COL1A1	17	48,260,650	48,278,993	3.6542	0.0149
	ENSG00000168542	COL3A1	2	189,839,046	189,877,472	4.8514	0.0498

Table 6.1. Group2; Top 10 Downregulated & Top 10 Upregulated genes in dataset.

	Ensembl Gene ID	Gene Name	Chr	Start	End	Fold Change	FDR (BH)
Downregulated	ENSG00000117152	RGS4	1	163,038,565	163,046,592	-13.5477	0.0003
	ENSG00000168542	COL3A1	2	189,839,046	189,877,472	-12.674	0.0026
	ENSG00000164161	HHIP	4	145,567,173	145,666,423	-10.7118	0.0007
	ENSG00000140937	CDH11	16	64,977,656	65,160,015	-9.1247	0.0006
	ENSG00000169851	PCDH7	4	30,722,037	31,144,728	-9.1034	2.33E-05
	ENSG00000166923	GREM1	15	33,010,175	33,026,870	-8.9563	0.001
	ENSG00000166033	HTRA1	10	124,221,041	124,274,424	-8.7589	0.0007
	ENSG00000115165	CYTIP	2	158,271,131	158,345,473	-8.2579	0.0003
	ENSG00000138650	PCDH10	4	134,070,470	134,129,356	-6.9348	0.004
	ENSG00000172572	PDE3A	12	20,522,179	20,837,315	-6.4175	7.58E-05
Upregulated	ENSG00000121858	TNFSF10	3	172,223,298	172,241,297	6.6386	0.0012
	ENSG00000013297	CLDN11	3	170,136,653	170,578,169	6.7213	0.0338
	ENSG00000137203	TFAP2A	6	10,393,419	10,419,892	6.8867	0.0043
	ENSG00000232810	TNF	6	31,543,344	31,546,113	7.0349	2.33E-05
	ENSG00000166920	C15orf48	15	45,722,727	45,740,959	7.4503	0.0002
	ENSG00000184292	TACSTD2	1	59,041,099	59,043,166	7.6042	0.0016
	ENSG00000163235	TGFA	2	70,674,412	70,781,325	7.9543	0.0002
	ENSG00000198910	L1CAM	X	153,126,969	153,174,677	9.1634	0.003
	ENSG00000169429	IL8	4	74,606,223	74,609,433	9.7168	0.016
	ENSG00000136244	IL6	7	22,765,503	22,771,621	16.5099	0.0012

Table 6.2. Group7; Top 10 Downregulated & Top 10 Upregulated genes in dataset.

Ensembl Gene ID	Gene Name	Chr	Start	End	Group 2 Fold Change	Group 2 FDR (BH)	Group 7 Fold Change	Group 7 FDR (BH)
ENSG00000253368	TRNP1	1	27,320,198	27,327,147	-2.5157	0.0143	2.0957	0.0267
ENSG00000158769	F11R	1	160,965,001	161,008,784	-2.3126	0.0152	2.4089	0.0064
ENSG00000196352	CD55	1	207,494,853	207,534,311	2.0724	0.0088	-2.3361	0.0013
ENSG00000136542	GALNT5	2	158,114,110	158,170,723	2.3666	0.0125	-4.0596	0.0002
ENSG00000197635	DPP4	2	162,848,751	162,931,052	-3.8738	0.0241	3.4487	0.0247
ENSG00000168542	COL3A1	2	189,839,046	189,877,472	4.8514	0.0498	-12.674	0.0026
ENSG00000183671	GPR1	2	207,040,040	207,082,771	2.3228	0.0237	-2.3854	0.0129
ENSG00000121858	TNFSF10	3	172,223,298	172,241,297	-3.1276	0.0363	6.6386	0.0012
ENSG00000185112	FAM43A	3	194,406,622	194,409,762	2.9923	0.0119	-3.2698	0.0036
ENSG00000157404	KIT	4	55,524,085	55,606,881	3.6289	0.0088	-3.3632	0.0054
ENSG00000038427	VCAN	5	82,767,284	82,878,122	-2.1506	0.0325	2.046	0.0335
ENSG00000113083	LOX	5	121,398,890	121,414,206	2.7761	0.0116	-4.4381	0.0004
ENSG00000160867	FGFR4	5	176,513,887	176,525,145	-2.3548	0.0099	2.0665	0.0129
ENSG00000146038	DCDC2	6	24,171,984	24,358,280	-3.8992	0.0055	2.6771	0.0174
ENSG00000106003	LFNG	7	2,552,163	2,568,811	-4.5124	0.0039	2.994	0.0131
ENSG00000086300	SNX10	7	26,331,541	26,413,949	-2.2183	0.0185	2.3581	0.0068
ENSG00000178445	GLDC	9	6,532,464	6,645,650	-2.9214	0.0371	2.7221	0.0405
ENSG00000151892	GFRA1	10	117,816,444	118,033,126	-3.5518	0.0072	3.2869	0.0048
ENSG00000165868	HSPA12A	10	118,430,703	118,502,085	-3.5456	0.0039	2.2208	0.0274
ENSG00000136052	SLC41A2	12	105,196,331	105,352,522	-2.0701	0.027	2.1182	0.0158
ENSG00000136153	LMO7	13	76,194,570	76,434,004	2.2247	0.0208	-2.2179	0.0128
ENSG00000100557	C14orf105	14	57,936,019	57,960,585	-3.2144	0.0056	2.0351	0.045
ENSG00000166145	SPINT1	15	41,136,216	41,150,405	-2.0184	0.0155	2.5389	0.0013
ENSG00000166147	FBN1	15	48,700,503	48,938,046	2.0706	0.0099	2.8223	0.0004
ENSG00000169594	BNC1	15	83,924,655	83,953,466	-2.8191	0.0183	2.4581	0.0231
ENSG00000153822	KCNJ16	17	68,071,426	68,131,744	-3.0141	0.0394	3.7244	0.0126
ENSG00000154864	PIEZO2	18	10,670,238	11,148,761	2.5537	0.0401	-3.307	0.0085
ENSG00000053747	LAMA3	18	21,269,562	21,535,030	-2.0468	0.0374	2.0982	0.0248
ENSG00000074181	NOTCH3	19	15,270,445	15,311,792	2.676	0.0447	-3.4295	0.0116
ENSG00000127528	KLF2	19	16,435,651	16,438,337	2.4707	0.0279	-2.54	0.0168
ENSG00000105699	LSR	19	35,739,559	35,758,865	-2.5724	0.0056	3.2561	0.0005
ENSG00000101017	CD40	20	44,746,911	44,758,502	-2.0112	0.0291	2.7626	0.002
ENSG00000188064	WNT7B	22	46,316,242	46,373,009	-2.3267	0.0149	2.0155	0.0234
ENSG00000198947	DMD	X	31,132,808	33,357,558	2.1093	0.0413	-3.7068	0.001
ENSG00000147027	TMEM47	X	34,645,181	34,675,405	3.1498	0.0132	2.4763	0.0268

Table 6.3. Genes dysregulated in both group2 & group7. Note that the expression change is in the opposite direction for many of the genes listed. Only FBN1 and TMEM47 show common upregulation in both groups.

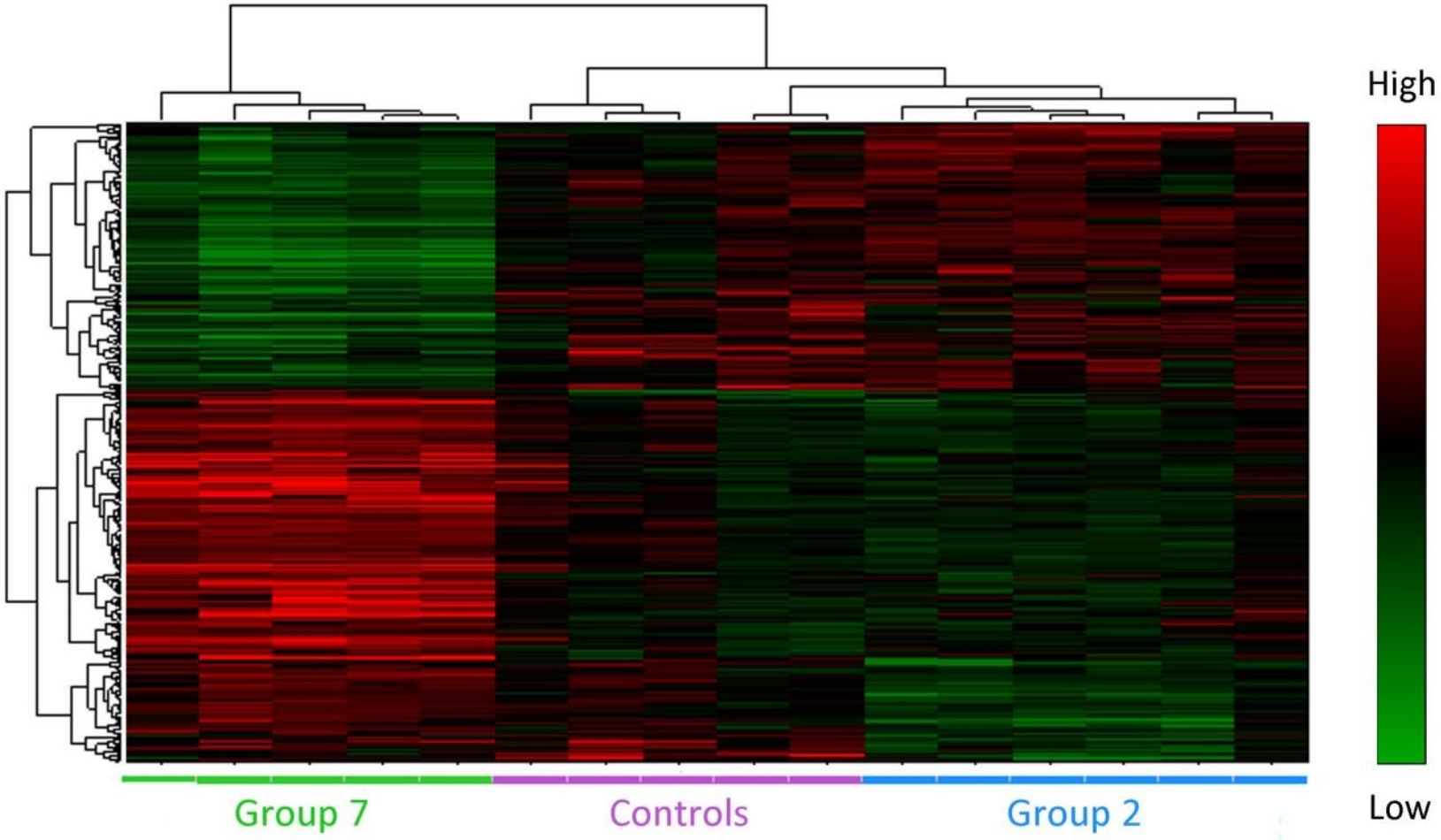


Figure 6.2. Heatmap displaying Log2 transformed RPKM expression values per sample. Only group 2, group 7, and controls are shown. Data is displayed for the 440 genes significantly dysregulated in group 2 and/or group 7.

A number of genes which are associated with, or are candidates for, CDH or lung development are identified as dysregulated in group7 and / or group 2. Interestingly, COL3A1 is downregulated in group7 (fold change -12.674, FDR 0.0026). Mutations in COL3A1 are associated with Ehlers Danlos syndrome in humans [OMIM #130050], in which CDH has been observed (Lin et al., 2006). LFNG is dysregulated in both groups (group7 fold change 2.994, FDR 0.0131; group2 fold change -4.5124, FDR 0.0039). LFNG is expressed in the pulmonary epithelium, and KO mice exhibit defects in impaired myofibroblast differentiation and alveogenesis [Xu et al. (2009)]. WNT7B is dysregulated in both groups (group7 fold change 2.0155, FDR 0.0234; group2 fold change -2.3267, FDR 0.0149) and was reported to be downregulated in the nitrofen induced rodent (Takayasu et al., 2007). Furthermore, the effects of Wnt7b on lung development were examined using a conditional Wnt7b-null mouse (Rajagopal et al., 2008), which revealed Wnt7b-null lungs were hypoplastic, yet displayed largely normal patterning and cell differentiation. In contrast to results from hypomorphic Wnt7b models, decreased replication of both developing epithelium and mesenchyme were seen, without abnormalities of vascular smooth muscle development. BNC1 is dysregulated in both groups (group7 fold change 2.4581, FDR 0.0231; group2 fold change -2.8191, FDR 0.0183). The BNC1 gene is in the 15q25.2 microdeletion region which has been recently associated with CDH, including in 2 cases we have identified (Chapter 4) (Wat et al., 2010). Taken together, this provides support for potential involvement of BNC1 in some cases of CDH pathogenesis. PDE3A was also found to be significantly downregulated in group7 (fold change -6.4175, FDR 7.72E-08). This is of potential interest given the investigation of sildenafil as a possible therapeutic treatment for CDH, via inhibition of PDE5A. PDE3A is also a target of sildenafil. Sildenafil has recently been shown to improve pulmonary vascular development in a rodent CDH model and in humans with CDH (Luong et al., 2011; Stultz et al., 2013). A number of genes dysregulated in group 7 are also observed to be associated with diaphragm abnormalities in mouse models (Tables 1.2.1 & 1.2.2, Chapter 1, this thesis). Downregulated genes include; LOX (fold change -4.4381, FDR 0.0004); DMD (fold change -3.7068, FDR 0.001); TCF21 (fold change -2.6663, FDR 0.0049); and TGFB1 (fold change -2.0195, FDR 0.0044). Upregulated genes include; PTPRD (fold change 2.3708, FDR 0.0405); and FBN1 (fold change 2.8223, FDR 0.0004).

Network & Pathway Analysis

Downstream Effects Analysis

Pathway analysis was performed of the dysregulated gene lists for each group individually using the IPA software application (Ingenuity Pathway Analysis, Ingenuity Systems, CA, USA). From this pathway analysis, predictions are also given for activation and inhibition of specific biological functions based upon the direction of expression change observed (see methodology). Table 6.4 provides details of the biological functions for group2 which are predicted to be increased or decreased. Interestingly, lung development is predicted enriched for with a p-value of 7.76E-05, but an activation z-score of only -0.962, which may suggest decreased lung development, but without clear significance. Nine molecules are annotated as involved in lung development and are significantly dysregulated in group2; COL3A1, FBN1, FGFR4, KLF2, LFNG, LOX, NOTCH3, PDGFB, WNT7B. Additionally, molecules significantly dysregulated in group2 and involved in CDH are FBN1 and LOX, although these genes are both upregulated.

From the pathway analysis performed using IPA on the dysregulated gene sets, Table 6.5 provides details of the biological functions which are predicted to be increased or decreased for group7 also. Interestingly, respiratory development (23 genes, p-value 1.39E-06, activation Z-score 2.896) and lung development (22 genes, p-value 5.95E-07, activation Z-score 2.722) are the functions predicted activated with highest significance. Epithelial-mesenchymal transition (EMT) (11 genes, p-value 2.76E-05, activation Z-score -2.530) and inflammation of lung (23 genes, p-value 1.10E-05, activation Z-score -2.444) are the functions predicted inhibited with highest significance. Vascularisation is also predicted to be decreased, with 18 genes whose expression change is consistent with decreased vascularization.

Functions Annotation	p-Value	Predicted Activation State	Activation z-score	Molecules	# Molecules
hypoplasia of organ	4.48E-03	Increased*	1.964	HS6ST2, LOX, LSR, PAX2, RASSF2, RPS6KA5, WNT7B	7
organismal death	5.79E-06	Increased*	1.860	AMOT, CD40, COL1A1, COL3A1, DMD, E2F8, FBN1, FGFR4, GFRA1, GUCY1B3, HIPK2, HNF1B, KIT, KLF2, KRT19, LAMA3, LAMA4, LFNG, LMO7, LOX, LSR, LTBP1, MLLT3, NES, NRK, OSR1, PAX2, PDGFB, PROS1, RASSF2, RPS6KA5, SPINT1, TNFSF10, UGT8, WNT7B	35
Growth Failure	7.54E-04	Increased*	1.772	AMOT, CREB3L1, E2F8, FGFR4, GUCY1B3, HIPK2, KLF2, NOTCH3, PDGFB, RASSF2, SPINT1, UGT8	12
vasculogenesis	1.97E-03	Decreased	-2.049	AMOT, CD40, COL1A1, F11R, HAS3, HS6ST2, KIT, KLF2, LOX, LTBP1, PDGFB, TNFSF10	12
proliferation of endothelial cells	8.00E-03	Decreased	-2.184	F11R, HAS3, KIT, KLF2, PDGFB, TNFSF10	6
proliferation of cells	2.03E-05	Decreased	-2.844	ABCB1, AKR1C1/AKR1C2, BNC1, CD40, CD55, COL1A1, CYP2S1, DPP4, E2F8, F11R, FBN1, FGFR4, GFRA1, GLDC, HAS3, HIPK2, HNF1B, HS6ST2, KCNJ16, KIT, KLF2, KRT19, LAMA3, LAMA4, LITAF, LOX, LTBP1, MLLT3, NES, NOTCH3, OSR1, PAX2, PDGFB, PTPN3, RPS6KA5, RTKN2, TNFSF10, TPD52, TYMS, VCAN, WNT7B	41

Table 6.4. Group 2; Biological functions which are predicted to be decreased or increased based upon the observed dysregulation in expression. We list those functions with Z-score >2, or<-2.
*No functions were predicted activated with a Z-score >2, so we list the top 3 activated functions and the respective Z-scores obtained.

Functions Annotation	p-Value	Predicted Activation State	Activation z-score	Molecules	# Molecules
respiratory system development	1.39E-06	Increased	2.896	BMP4, CDKN1C, COL3A1, CTGF, FBN1, FGF1, FGFR4, FOXA1, GREM1, IL6, ITGB6, KLF2, LFNG, LOX, NOTCH3, PCSK5, TCF21, TGFB1, TGFBR2, THBS1, TLR4, TNF, WNT7B	23
lung development	5.95E-07	Increased	2.722	BMP4, CDKN1C, COL3A1, CTGF, FBN1, FGF1, FGFR4, FOXA1, GREM1, IL6, ITGB6, KLF2, LFNG, LOX, NOTCH3, TCF21, TGFB1, TGFBR2, THBS1, TLR4, TNF, WNT7B	22
infiltration of cells	4.87E-11	Increased	2.516	ADAM8, AKR1B1, CD40, CFH, CTGF, CXCL16, CXCL2, CXCL5, F2R, F2RL1, F3, FST, HAVCR1, HMOX1, IL32, IL6, IL8, IRF6, ITGA4, KITLG, LDLR, MCAM, NFKBIA, NFKBIZ, PLA2G4A, PLAT, PPT2, STC1, TGFB1, TGFBR2, THBS1, TLR4, TNF	33
infiltration by neutrophils	3.53E-08	Increased	2.428	ADAM8, CFH, CTGF, CXCL2, CXCL5, F2RL1, HAVCR1, HMOX1, IL6, IL8, IRF6, NFKBIA, PLAT, TGFBR2, TLR4, TNF	16
cell movement of neutrophils	1.18E-06	Increased	2.283	ADAM8, CFH, CTGF, CXCL2, CXCL5, EDNRA, F2RL1, HAVCR1, HMOX1, IL6, IL8, IRF6, ITGA4, LYN, NFKBIA, PLAT, TGFB1, TGFBR2, TLR4, TNF, VEGFC	21
activation of phagocytes	1.04E-06	Increased	2.248	CD40, CFH, CLEC4E, CXCL5, DPP4, F2RL1, HAVCR1, HMOX1, IL6, IL8, IRF6, ITGB6, KIT, KITLG, LYN, RAPGEF3, SCN9A, TGFB1,	22

				THBS1, TLR4, TNF, TNFSF10	
quantity of neurons	1.11E-05	Increased	2.220	BMP4, CCND2, DLX2, EMX2, FST, GDNF, GFRA1, HOXB3, INHBA, JAG1, KCNIP3, L1CAM, LFNG, NEFH, NEFM, NR2F1, TFAP2A, TGFA, WNT7B	19
infiltration of blood cells	8.67E-10	Increased	2.189	ADAM8, AKR1B1, CFH, CTGF, CXCL16, CXCL2, CXCL5, F2R, F2RL1, F3, HAVCR1, HMOX1, IL6, IL8, IRF6, ITGA4, KITLG, LDLR, MCAM, NFKBIA, NFKBIZ, PLA2G4A, PLAT, PPT2, STC1, TGFB1, TGFBR2, THBS1, TLR4, TNF	30
function of blood cells	3.75E-05	Increased	2.105	ANPEP, CD40, CHST2, CLEC4E, CTSS, F11R, F3, GJA1, GPR68, HAVCR1, ICA1, IFI30, IL6, IL8, KCNN4, KIT, KLF2, LPAR2, LYN, MALT1, NFKBIA, NFKBIZ, PLA2G4A, PLAT, PTGS1, SEPT5, SPNS2, TGFB1, TLR4, TNF, TNFSF10	31
infiltration of granulocytes	6.69E-07	Increased	2.086	ADAM8, CFH, CTGF, CXCL2, CXCL5, F2R, F2RL1, HAVCR1, HMOX1, IL6, IL8, IRF6, NFKBIA, PLAT, TGFBR2, TLR4, TNF	17
cell spreading	1.95E-09	Increased	2.054	CD40, CDKN2A, EFNA1, EFNB1, EGF, FBN1, IL8, ITGA11, ITGA4, ITGB6, KITLG, L1CAM, LAMA3, LYN, MAP2, PAK3, PMP22, POSTN, RAPGEF3, SDC1, SYNM, THBS1, TNF	23
infiltration of leukocytes	3.44E-09	Increased	2.051	ADAM8, CFH, CTGF, CXCL16, CXCL2, CXCL5, F2R, F2RL1, F3, HAVCR1, HMOX1, IL6, IL8, IRF6, ITGA4, KITLG, LDLR, MCAM, NFKBIA, NFKBIZ, PLA2G4A, PLAT, PPT2, STC1, TGFB1, TGFBR2, THBS1, TLR4, TNF	29
activation of antigen presenting cells	1.19E-05	Increased	2.044	CD40, CLEC4E, CTSS, CXCL5, F2RL1, HAVCR1, IL6, IL8, ITGB6, LYN, RAPGEF3, SCN9A, TGFB1, THBS1, TLR4, TNF, TNFSF10	17
activation of dendritic cells	3.28E-05	Increased	2.035	CD40, F2RL1, IL6, IL8, LYN, SCN9A, TGFB1, TLR4, TNF, TNFSF10	10
invasion of cells	9.92E-17	Increased	2.000	ADAM8, ADAMTS15, ANPEP, BMP4, CDK14, CDKN2A, CTGF, CTSS, DPP4, EGF, ELF3, EPCAM, F2R, F3, FGFR4, FOXA1, FST, GDNF, GFRA1, GJA1, HAS2, HMOX1, IL6, IL8, INHBA, ITGA4, ITGB4, ITGB6, JUP, KITLG, L1CAM, LAMA3, LYN, MCAM, MMP15, MMP16, NFKBIA, PAK3, PLAT, POSTN, RGS4, SDC1, SDC2, SOD2, TFAP2A, TGFA, TGFB1, TGFBR2, THBS1, TNF, TNFSF10, UNC5C, VEGFC	53
accumulation of extracellular matrix	3.41E-06	Decreased	-2.000	ADAMTS5, CTGF, EDNRA, GREM1, TGFB1	5
neuritogenesis	1.53E-06	Decreased	-2.072	ATP8A2, DLX2, DNER, DPYSL3, EDNRA, EFNA1, EFNB1, GDNF, GFRA1, GJA1, IL6, KCNJ2, L1CAM, LAMA1, LOX, LYN, NEFH, NEFM, NFIA, NFKBIA, PALM, PDGFRB, PLAT, PMP22, SDC2, STX3, TGFB1, TNF, WNT7B	29
vascularization	7.01E-07	Decreased	-2.150	ANPEP, CD40, CTGF, EFNA1, EFNB1, EGF, FGF1, INHBA, ITGA4, KIT, PLA2G4A, STC1, TGFA, TGFB1, TGFBR2, THBS1, TNF, VEGFC	18
neovascularization of organ	3.88E-07	Decreased	-2.157	ANPEP, CTGF, EGF, FGF1, INHBA, KIT, PLA2G4A, TGFA, THBS1, TNF, VEGFC	11
glomerulosclerosis	4.03E-05	Decreased	-2.157	GDNF, HAVCR1, HMOX1, ITGB4, KCNN4, LYN, MTSS1, PDGFRB, STC1, TGFB1, TLR4	11
formation of plasma membrane projections	5.68E-07	Decreased	-2.237	ATP8A2, DLX2, DNER, DPYSL3, EDNRA, EFNA1, EFNB1, EGF, GDNF, GFRA1, GJA1, IL6, KCNJ2, KITLG, L1CAM, LAMA1, LOX, LYN, MAP2, NEFH, NEFM, NFIA, NFKBIA,	32

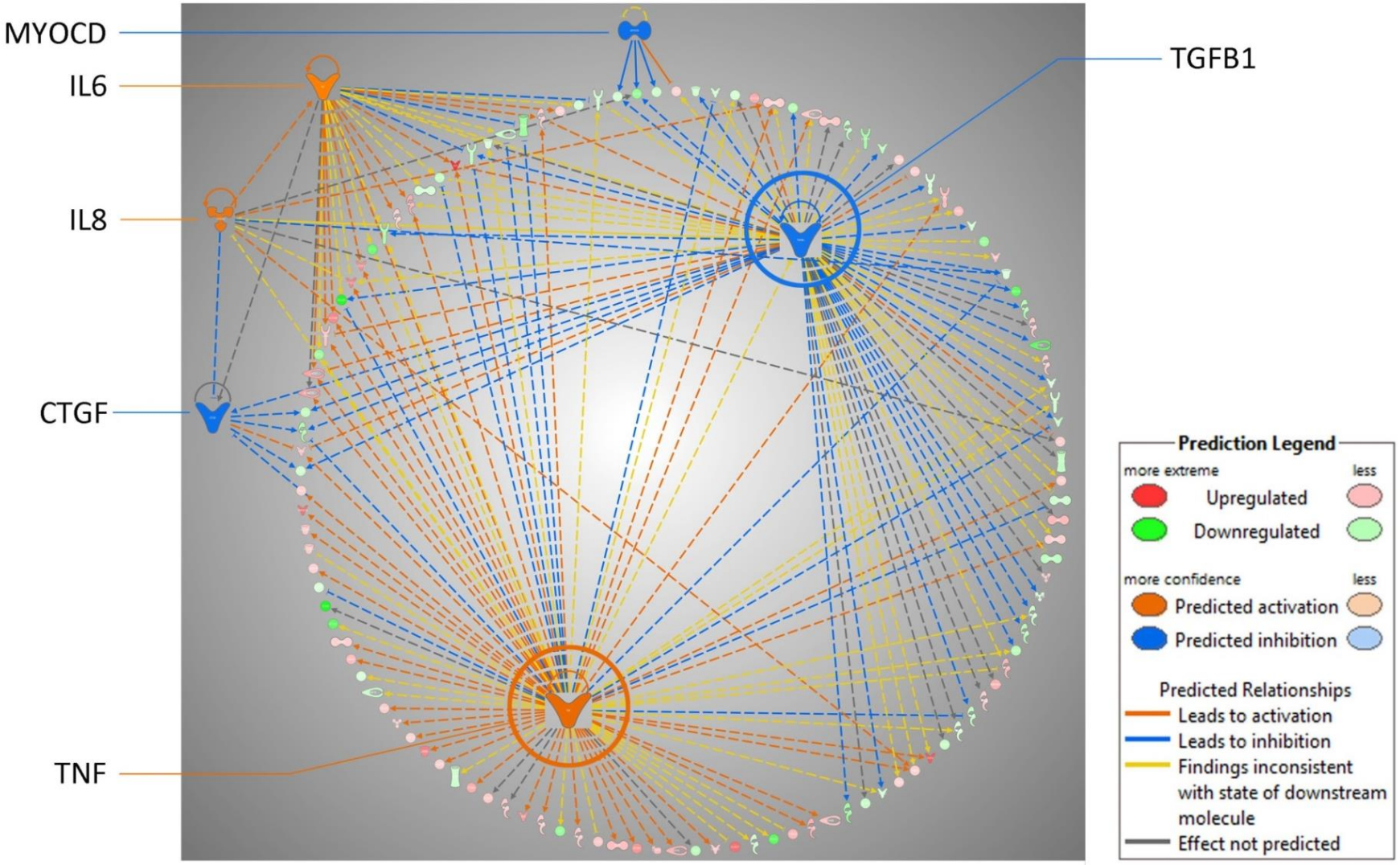
				PALM, PDGFRB, PLAT, PMP22, SDC2, STX3, TGFB1, TNF, WNT7B	
neovascularization	2.37E-08	Decreased	-2.286	ANPEP, CD40, CTGF, EFNA1, EGF, FGF1, INHBA, ITGA4, KIT, PLA2G4A, TGFA, TGFB1, TGFBR2, THBS1, TNF, VEGFC	16
Neurodegeneration	1.66E-05	Decreased	-2.351	FBXO2, FGF1, GDNF, GPR37, IL6, NEFH, PLAT, PPT2, SEPT5, SOD2, TGFA, TGFB1, TGFBR2, TLR4, TNF	15
inflammation of lung	1.10E-05	Decreased	-2.444	CDH11, CTSS, EDNRA, ELF3, F2RL1, FBN1, HMOX1, IL6, IL8, INSIG1, ITGB6, KIT, LDLR, LPAR2, LYN, PDGFRB, PLA2G4A, PLAT, PTGS1, TGFB1, THBS1, TLR4, TNF	23
epithelial-mesenchymal transition	2.76E-05	Decreased	-2.530	EFNA1, EGF, ESRP2, FGF1, HAS2, JAG1, NFKBIA, POSTN, TGFB1, TGFBR2, TNF	11

Table 6.5. Group 7; Biological functions which are predicted to be decreased or increased based upon the observed dysregulation in expression. We list those functions with Z-score >2, or<-2.

Upstream Regulator and Mechanistic Network Analysis

The mechanistic network analysis for group7 predicts TGFB1 inhibition and TNF activation as being largely responsible for the majority of the downstream dysregulation observed. The highest p-values observed for predicted upstream regulators which were concordant with the observed dysregulation in gene expression from group 7 were; TNF (p-value 7.99E-22), predicted activated; and, TGFB1 (p-value 3.31E-17), predicted inhibited. TGFB1 is predicted to be inhibited (activation Z-score -1.774; p-value 3.31E-17), and shows a fold change of -2.02 (FDR 0.0044). CTGF is predicted to be inhibited in group7 (activation Z-score -2.000; p-value 1.69E-02), and shows a fold change of -3.54 (FDR 2.00E-04). MYOCD is predicted to be inhibited in group 7 (activation Z-score -1.925; p-value 8.63E-03), and is downregulated (fold change -2.9819, FDR 0.0038). Tumor necrosis factor (TNF) is a multifunctional proinflammatory cytokine which is predicted activated in group 7 (activation Z-score 3.358; p-value 7.99E-22), and is observed to be upregulated (fold change 7.0349, FDR 2.33E-05). IL6 is predicted activated, but without clear significance, in group 7 (activation Z-score 0.673; p-value 6.23E-07), and is upregulated (fold change 16.5099, FDR 0.0012). IL8 is also upregulated (fold change 9.7168, FDR 0.016). It is clear from the network displayed in Figure 6.3 that TGFB1 downregulation is generally causing a downstream inhibitory effect, and that conversely the upregulation of TNF is causing activation of many downstream target genes.

Figure 6.3 (following page). A 'mechanistic network' analysis was performed using IPA (Ingenuity Systems). Significantly dysregulated genes which were predicted to be activated or inhibited (Z-scores <-2, >2) were selected and added to the same network. The network displayed shows the downstream effects of a number of key genes including; TGFB1, TNF, CTGF, IL6, IL8, and MYOCD. The orange arrows indicate predicted activation and a concordant upregulation in expression of the target gene. The blue arrows indicate predicted inhibition and a concordant downregulation of the target gene. The yellow arrows indicate discordance for the expected to observed expression change. Molecules in red are upregulated, those in green are downregulated, with the colour intensity reflecting the size of the fold change.


MYOCD
IL6
IL8
CTGF
TNF
TGFB1
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted

Discussion

Amniotic fluid (AF) cells are an available source of foetal material during pregnancy and we identify different classes of expression profiles in foetuses affected with isolated CDH. Goumy *et al* previously studied foetal skin fibroblasts from CDH foetuses as a model to investigate CDH (Goumy et al., 2010). However, these foetal samples are not so readily available. The authors used targeted expression analysis in CDH patients which revealed altered levels of RARs, RALDH2, and CYP26 in two of seven foetuses investigated. We do not observe any dysregulation in key genes involved in the RA pathway and considered to be involved in CDH pathogenesis such as STRA6, RARs, NR2F2, GATA4, or ZFPM2. However, we do observe downregulation of NR2F1 in group 7 (fold change -5.4386, FDR 0.0003) which like NR2F2 is a member of the steroid/thyroid hormone receptor superfamily. NR2F1 & 2 share similar characteristics interacting with other members of the steroid/thyroid hormone receptor superfamily such as TR, RAR, RXR and VDR, which play important roles in regulation of gene expression during development, differentiation and homeostasis. NR2F1 and 2 are orphan members of this superfamily of nuclear receptors and have been shown to negatively regulate the ability of these nuclear receptors to regulate target gene transcription. Two different mechanisms are proposed to regulate this repression; NR2F1 & 2 bind to AGGTCA direct repeats, including response elements for TR, RAR, RXR and VDR, which permits competition for binding to response elements; and NR2F1 & 2 heterodimerise with RXRs, which are essential cofactors for binding of RARs, TRs and VDR to their respective response elements. Detection of COUP-TF transcripts during mouse development reveal discrete spatial and temporal expression domains consistent with COUP-TFs being involved in regulation of gene expression during embryogenesis. Some of these expression domains co-localize with those of TR, RAR, and RXR. The simultaneous expression of these genes raise the possibility that NR2F1 & 2 act as negative regulatory factors during development and differentiation (Zhou et al., 1999; Qiu et al., 1997; Pereira et al., 1995; Qiu et al., 1994).

From our network analysis of gene expression changes in group 7, we observe that TGFB1 is predicted to be inhibited (activation Z-score -1.774; p-value 3.31E-17), and shows a fold change of -2.02 (FDR 0.0044). TGF-β1 null mice develop apparently normally, but soon die from aggressive pulmonary or gut inflammation due to a failure to negatively modulate the immune system (McLennan et al., 2000). Disruption of TGF-β signalling in TAB1 null mice results in lethal cardiovascular and lung abnormalities (Komatsu et al., 2002). Abnormal diaphragm morphology has also been reported in a Tgfb1 mouse model; the diaphragm

showed inflammation and necrosis which were severe enough to interfere with normal respiration (Shull et al., 1992). CTGF is predicted to be inhibited in group 7 (activation Z-score -2.000; p-value 1.69E-02), and shows a fold change of -3.54 (FDR 2.00E-04). CTGF is upregulated in group2 (fold change 1.8746, FDR 0.0317). Expression array analysis identified CTGF as a novel gene downregulated in the nitrofen rodent model of CDH (Mesas-Burgos et al., 2010b). CTGF was shown to be upregulated in the *hyperplastic* lungs of a rodent model following tracheal ligation (Mesas-Burgos et al., 2009). The same group subsequently investigated the expression pattern of CTGF during lung development (Mesas-Burgos et al., 2010a) demonstrating reduced CTGF protein levels in the nitrofen rodent model of CDH. Interestingly, the reduced pulmonary gene expression of CTGF observed in the nitrofen CDH rodent model was reversed after prenatal RA treatment, which may support lung growth by promoting alveologenesis in the nitrofen-induced CDH model (Ruttenstock et al., 2011b). MYOCD is predicted to be inhibited in group 7 (activation Z-score -1.925; p-value 8.63E-03), and is downregulated (fold change -2.9819, FDR 0.0038). Recently, it was shown that knockdown of MYOCD was sufficient to induce vascular smooth muscle cell proliferation (Pfisterer et al., 2012).

From our analysis, we identify 2 classes of isolated CDH foetuses based upon the expression profiles of cultured AF cells. However, these expression profiles do not confidently correlate with clinical features including; left-sided CDH vs right-sided CDH; severity of herniation; or clinical outcome, Table 6.6.

Table 6.6 (following page). Clinical information concerning those isolated CDH patients analysed in this study. Groups refer to those classes of foetuses with similar expression profiles, where information for group 2 and group 7 is presented and discussed in the text. Group 1 contains only 2 foetuses and was not analysed further. Groups 4, 5, and 6 showed similar expression profiles to the control group. Group 8 contains 2 outliers from those in group 7.

groups	CDH type	Sex	GA at AF sample	OE LHR	LHR	FETO y/n	Increase in OE LHR / %	Increase in LHR / %	MRI OE TFLV	LIVER up/down	Poly hydramnios	GA at delivery	Neonatal death <1d	Neonatal death <7d	Survival till discharge	PH refractory to NO
1	left	M	27		0.7	y		71	30	up	y	38.6	n	y	n	
1	left	M	29		0.99	y		62	37	up	y					
2	right	M	29	27.2	0.7	y	338	386	19	up	y		n	n	y	n
2	right	M	26	28.7		y	205		26	up	n	31.7	n	n	n	y
2	left	M	27		0.81	y		260		up	n		n	n	y	
2	left	M	27	21.9		y	169		31	up	y	37.0	n	n	y	n
2	right	UNK	28		0.93	y		115	33	up	n	37.4	n	n	y	n
2	right	M	29	29.7		y	190		16	up	y		n	n	y	
4	right	UNK	29		0.62	y				up	y	34.7	y	y	n	
5	left	M	29	20		y	85		25	up	n	31.9	n	n	y	n
5	left	M	28	8.3		y	8		7	up	y	36.6	y	y	n	
6	right	UNK	27	22		y	123		13	up	n	36.6	n	n	y	n
6	right	M	28	34		y	97		29	up	n	37.7	n	n	y	n
6	right	UNK	27	20.6		y			35	down	n	29.0	y	y	n	
6	left	M	26		0.74	y		130		up	n	35.0	y	y	n	
6	left	M	26		0.5	y		438		up	y	36.4				
7	left	M	25	22	0.8	y	209	263	19	up	y	31.4	n	n	y	n
7	left	M	28	20	0.79	y	80	103	14	up	y	32.1	n	n	y	n
7	left	M	25	32.3		n			38	up	n	38.4	n	n	y	n
7	left	M	27		0.8	y		94	22	up	n		y	y	n	
7	right	UNK	28	45	1.2	y	62	75	38	up	y	36.7	y	y	n	y
8	left	M	28	33		y	37		24	up	n	32.9	y	y	n	
8	left	M	27	32		n			29	up	n	35.7	y	y	N	

The expression changes we observe are suggestive of disruption to a common underlying genetic network, or perhaps a similar biological response to disease for each group. While these changes may not correlate with a predictive clinical feature this information suggests that for one group (group7) of isolated CDH patients, representing 20-25% of patients in this study, downregulation of TGFB1, CTGF, and MYOCD, as well as upregulation of TNF and IL6, may be important factors contributing to the diaphragm and / or lung pathogenesis. It is uncertain whether the changes seen are related to the causes of CDH, or are merely the effects of the herniation and the hypoplastic lungs and associated anomalies. However, there exist 2 molecular subtypes which may suggest a common underlying genetic defect or similar progression of disease for these groups.

The largest list of significantly dysregulated genes was identified from group 7, which can be summarized as having; increased activation of genes and pathways linked to respiratory development; and inhibition of genes and pathways associated with epithelial-mesenchymal transition (EMT), and with vascularization. Furthermore, decreased expression of TGFB1, and increased expression of TNF, can explain a large proportion of the downstream dysregulation in gene expression observed. While a lower number of genes are observed to be significantly dysregulated in group2, this group is characterized by inhibition genes and pathways involved in cell proliferation, proliferation of endothelial cells, and vasculogenesis, and by activation of genes and pathways involved in hypoplasia of organ, organismal death, and growth failure. The finding of commonly dysregulated genes between 2 molecular subtypes identified, but with the opposing direction of expression change, points towards to dosage sensitive genes which may cause, or be a response to, the progression of CDH and the associated lung hypoplasia.

Currently, tracheal occlusion during pregnancy is the only fetal intervention for moderate-severe cases of isolated CDH (Dekoninck et al., 2011). While this is effective in treating the pulmonary hypoplasia in the prenatal phase, the pulmonary hypertension observed after birth is still a major factor contributing to the mortality and morbidity in survivors (Danzer et al., 2008). Therapeutic studies using steroids in lamb models of CDH have demonstrated improvements in pulmonary vascular remodeling (Davey et al., 2007; Davey et al., 2006b; Davey et al., 2006a). In a rodent model of CDH reduced VEGFR1 and VEGFR2 expression was observed, which were subsequently increased by the use of steroids in conjunction with tracheal occlusion (Schmidt et al., 2010). Targeted therapies already proposed to combat pulmonary hypertension may also prove beneficial to block key prenatal inflammatory

signals which may benefit a subset of foetuses with CDH thus complementing the current foetal therapy (O'Callaghan et al., 2011). This is of potential interest given the apparent pro-inflammatory response observed in group 7, with upregulation of TNF, IL6 and IL8. Increased levels of TNFα, IL6, and IL1 have been previously described in patients with primary pulmonary hypertension (Humbert et al., 1995; Soon et al., 2010). Interestingly, Schaible et al have reported increased concentrations of cytokines in newborns with CDH including; TNFα, IFNα, IL3, IL6, IL7, IL8, IL10, CCL3, and CCL4). The authors propose that CDH is associated with a pulmonary inflammatory response immediately after birth (Schaible et al., 2011).

Vuletin et al assessed the potential of prenatal predictors of postnatal severe pulmonary artery hypertension (PAH) in isolated left congenital diaphragmatic hernia (CDH), defining a new prenatal pulmonary hypertension index (PPHI) (Vuletin et al., 2010). Patients with systemic/suprasystemic and subsystemic pulmonary hypertension at 3 weeks of age were identified. Diameters of the right pulmonary artery, left pulmonary artery (LPA(d)), aorta, and the length of vermis of the cerebellum were obtained from prenatal magnetic resonance imaging to calculate the PPHI [=(LPA(d)/length of vermis of the cerebellum) x 10] and the modified McGoon index (MGI) [=(diameter of the right pulmonary artery + LPA(d))/diameter of aorta]. PPHI and MGI were compared with LHR, percent predicted lung volume, and total lung volume for pulmonary hypertension and survival. The PPHI and MGI had a significant, negative correlation with pulmonary hypertension. The PPHI and MGI were significantly lower in the systemic/suprasystemic PAH group compared with the subsystemic PAH group, with no significant differences between the groups comparing the LHR, percent predicted lung volume, and total lung volume. The authors conclude that both PPHI and MGI accurately predict the severity of postnatal PAH in isolated left CDH (Vuletin et al., 2010).

Inflammatory markers can also be an indication of intra-amniotic infections such as chorioamnionitis (inflammation of the fetal membranes due to a bacterial infection). It has been demonstrated in a mouse model that inflammatory mediators can cause dysfunction of the amniotic barrier by disruption of amniotic tight junctions (TJs) (Kobayashi et al., 2010). Injection of IL6 into the amniotic cavity induced disruption of amniotic tight junctions by decreasing claudin3 and claudin4 levels at the apical junction, and injection of TNFα weakened the amniotic barrier by inducing apoptosis of amniotic epithelial cells. Intrauterine inflammation has also been associated to preterm delivery, which may be of

relevance given the high degree of preterm premature rupture of membranes (PPROM) following prenatal therapy by FETO which impacts gestational age at delivery and complicates balloon removal prior to birth (Deprest et al., 2011). Significantly higher levels of pro-inflammatory cytokines including TNFα, IL6 and IL8 have been reported in association with preterm delivery (Veleminsky, Jr. et al., 2008; Zhang et al., 2000; La Sala et al., 2012), though there is some discordance between different studies (Puchner et al., 2012; Weissenbacher et al., 2013).

Previous studies of the origin of amniotic fluid derived cells are limited mainly to morphological and culture characteristics (Gosden, 1983; Tyden et al., 1981). Given that cells derived from AF are a mixture of different cell types we cannot fully exclude that the changes we observe are not differences in gene expression *per se* but represent differences in the cellular composition. Culture of cells is also a factor which may influence gene expression. Recently, several groups have focused on global gene expression analysis of cell-free RNA in AF supernatant including for studies of trisomies 21 and 18 (Hui et al., 2012b; Hui et al., 2013; Hui et al., 2012a; Koide et al., 2011). This may represent an alternative source of foetal material for investigation. However, based upon our analyses and limited prior observations of apparent inflammatory responses in CDH patients which may contribute to severity of pulmonary hypertension, we propose that a follow up study at the protein level using AF supernatant or cord blood in a large cohort of foetuses affected with isolated CDH may provide more conclusive evidence to identify potential therapeutic targets in a subset of patients.

We do not identify dysregulation of key genes involved in the retinoic acid (RA) pathway. While much data has been generated by targeted gene expression analysis in models of CDH (reviewed in (Brady et al., 2010), there is little from unbiased genome-wide analyses. Many studies have utilized the nitrofen CDH rodent model, with a number of these also demonstrating 'rescue' of the defect by supplementary RA (Montedonico et al., 2006; Montedonico et al., 2008; Doi et al., 2009; Ruttenstock et al., 2012; Schmidt et al., 2012; Pederiva et al., 2012; Doi et al., 2011b; Ruttenstock et al., 2011b; Doi et al., 2011a; Ruttenstock et al., 2011c; Ruttenstock et al., 2011a; Doi et al., 2010c; Doi et al., 2010b; Doi et al., 2010a; Ruttenstock et al., 2010). The molecular relevance of the nitrofen model to the human situation is questionable, and the dietary supplementation with RA is also controversial, particularly in the absence of an identifiable defect within this pathway. Interestingly, Beurskens et al reported that CDH is strongly associated with low retinol and

RBP levels in newborns, independent of maternal retinol status, supporting the retinoid hypothesis (Beurskens et al., 2010). More recently, the same group reported that reduced dietary vitamin A intake during pregnancy was significantly associated with an increased risk of CDH in the offspring (Beurskens et al., 2013). These studies have been conducted on small sample cohorts and further investigation in larger study populations is still necessary. Altered levels of RA due to common variants in genes involved in the retinoid signalling pathway have also been shown (El et al., 2010; Manolescu et al., 2010).

In light of our results using an unbiased approach and observations from several recent targeted studies which have also found upregulation of inflammatory molecules, it would be of interest to examine protein levels in AF supernatant in a larger cohort of moderate-severe isolated CDH patients to determine if this can confirm a subset of foetuses with a pro-inflammatory response which may represent a novel therapeutic target complementing the current FETO therapy.

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Chapter 7

Discussion and Future Perspectives

Chapter 7. Conclusions & Future Perspectives

7.1 Prenatal Diagnosis

7.1.1. Chromosomal Microarrays for Prenatal Diagnosis

The general consensus is growing that chromosomal microarrays can be implemented in the prenatal setting as the first tier test (Shaffer et al., 2012; Wapner et al., 2012b; Hillman et al., 2013; Callaway et al., 2013; Brady et al., 2013b). Those arrays do provide a higher diagnostic yield, detecting submicroscopic CNVs undetectable by conventional karyotyping, as well as larger genomic imbalances, and within a faster timeframe. In the first part of this thesis (Chapter 3) we demonstrated the added value of chromosomal microarray analysis for prenatal diagnosis in the presence of ultrasound abnormalities. Our findings demonstrated an increase in the diagnostic yield of pathogenic CNVs of 6.5 % in comparison to conventional karyotype alone, with 2.6 % of those CNVs detected being submicroscopic and completely undetectable by karyotype. A meta-analysis of prenatal microarray studies reported an increased diagnostic yield of chromosomal microarrays over karyotyping of 10% (8-13%, 95% confidence interval) in the presence of ultrasound anomalies (Hillman et al., 2013). More recently, a literature review of prenatal microarray studies estimated a 7% increase in the diagnostic yield in the presence of ultrasound anomalies (Callaway et al., 2013). The largest prospective studies in recent years report increased detection rates of pathogenic CNVs of between 6-8% in the presence of ultrasound anomalies (Shaffer et al., 2012; Breman et al., 2012; Wapner et al., 2012b). We summarise the findings of these and other recent prenatal array studies in Table 7.1.1 (Wapner et al., 2012b; Shaffer et al., 2012; Breman et al., 2012; Lee et al., 2012; Fiorentino et al., 2011; Armengol et al., 2012; Park et al., 2011; Hillman et al., 2011). Using the results from these studies the increased detection rate of pathogenic CNVs above karyotyping alone is calculated to be ~2.1% regardless of referral indication, rising to ~5.3% where the referral was due to abnormal ultrasound findings. Arrays increased the detection rate by 0.5-1.7% for lower-risk referral indications such as advanced age or abnormal biochemical screen, with no abnormal ultrasound findings and a normal karyotype. Lee et al also estimated the baseline risk of a pathogenic submicroscopic CNV to be ~0.52% (Lee et al., 2012).

Total No. of cases	Overall Detection Rate[1]	Detection Rate Above karyotyping			VOUS	Clinically significant but unrelated to phenotype	Study
		Abnormal US	AMA, high-risk biochemical screening	Any indication			
4282[2] 4340 total, 58 mosaic excluded	11.7 (8.7%)[3] (0.5%)[4]	6.0%	1.7% AMA 1.6% screening	2.5%	1.5%	1.3%[5] normal karyotype 3.6% abnormal US 0.8% normal US	(Wapner et al., 2012b)
3876[2]	NR	6.5% (7.6%)[6]	NR	5.3% (6.5%)[6]	4.2% (0.39%)[7]	1.3%	(Shaffer et al., 2012)
1075[2]	7.6% total (4.2 % excluding known abnormals)	NR	NR	1.7%	1.6%	NR	(Breman et al., 2012)
3171	2.7%	NR	NR	1.1%	0.6% (0.2%)[7]	0.52%[8]	(Lee et al., 2012)
1037	3.3%	NR	NR	0.9%	0%	NR	(Fiorentino et al., 2011)
906	6.2%	3.5%	1.1% AMA 0.4% screening 1.7% Anxiety	1.6%	1.9%	NR	(Armengol et al., 2012)
4073	1.8%	NR	NR	0.27%	NR	NR	(Park et al., 2011)
751[9]	NR	5.2%	NR	3.6%	1.1%	NR	(Hillman et al., 2011)
AVERAGE TOTALS		**5.30%**		**2.12%**	**1.56% (0.96%)[10]**		

Table 7.1.1. Summarises the results from the most recent chromosomal microarray studies applied for prenatal diagnosis.

US = ultrasound; AMA = advanced maternal age; VOUS = variant of uncertain significance; NR = not reported.

[1] Including karyotypically visible abnormalities for some studies; [2] Number of 'unbiased' cases used for calculations; [3] Automosomal or sex chromosome aneuploidies; [4] Unbalanced rearrangements or marker chromosomes; [5] Recurrent CNVs associated with autism and neurocognitive alterations; [6] Detection rates for oligonucleotide-based arrays only; [7] Percentage of VOUS occurring de novo; [8] Baseline risk calculation for pathogenic CNV in pregnancies with no sonographic anomalies; [9] 10 studies included, 8 studies used for meta-analysis; [10] Using de novo VOUS values for calculation.

Furthermore, arrays provide the ability to detect a number of microdeletion / microduplication syndromes, some of which may cause severe childhood developmental disorders but may not show any foetal abnormality by classic ultrasound techniques. In the absence of ultrasound abnormalities, chromosomal microarrays were reported to increase the detection rate above karyotyping by 1.7% in cases of advanced maternal age, and 1.6% for those with abnormal biochemical screen (Wapner et al., 2012b). Another study reported rates of 1.1% for advanced maternal age, 0.4% from abnormal biochemical screening, and 1.7% for referral due to maternal anxiety (Armengol et al., 2012). The National Institute of Child Health and Human Development study (NICHD, USA) reported clinically significant findings unrelated to the initial referral reason in 1.3% with normal karyotype, 3.6% with abnormal ultrasound features, and 0.8% where ultrasound findings were normal. Shaffer et al report a similar rate of 1.3% (Shaffer et al., 2012), and Lee et al estimate the baseline risk at 0.52% (Lee et al., 2012). Given these findings the use of chromosomal microarrays as the first tier test for all invasive prenatal referrals seems warranted.

In the future, routine prenatal screening by chromosomal microarray analysis will most likely be offered to any pregnant women who undergo an invasive procedure given the faster reporting time and the mainstream use of arrays for postnatal diagnosis. In Belgium, chromosomal microarray analysis has recently been recommended as the primary prenatal diagnostic test following invasive sampling (see the Belgian Society of Human Genetics (BeSHG) Summary Guidelines for Prenatal Chromosomal Microarray Analysis and Genetic Counselling for further reference); [http://www.beshg.be/download/annex_2_summary_of_array_and_prenatal_guidelines_20130502.pdf].

Ethical concerns have long been hampering the routine introduction of chromosomal microarrays for prenatal diagnosis (Novelli et al., 2013; McGillivray et al., 2012; Beaudet, 2010). There was (and still is) concern about how to deal with unsought for findings, late onset disorders, CNVs with variable expressivity and/or penetrance, and variants with only mild phenotypic anomalies. Variants of uncertain significance encompass a spectrum of findings which can in most cases be sub-classified as likely pathogenic, likely benign or truly of unknown significance based upon factors such as size, gene content, inheritance status, and the presence of overlapping CNVs in low numbers of patients or controls (Vermeesch et al., 2012). Current experience estimates the number of cases where difficulties in counselling arise due to the detection of VOUS and risk factors are limited to 1-2% (Shaffer

et al., 2012; Wapner et al., 2012b; Hillman et al., 2013; Brady et al., 2013b). Nevertheless, more research and consensus guidelines in this area would help both clinicians and families.

The penetrance risks for a number of recurrent CNVs have been estimated based upon the frequencies in patients and controls (Kaminsky et al., 2011; Cooper et al., 2011; Rosenfeld et al., 2013). However, while it is possible to calculate a population-based risk, it is impossible in the prenatal setting to predict the phenotypic outcome in the future individual. Thus, in the prenatal setting and in the absence of phenotypic anomalies, classification of a CNV as pathogenic where evidence exists for incomplete penetrance is questionable. The current approach we advocate for the reporting of risk factors and for which there is now a consensus agreement by the 8 regional genetics centres in Belgium, is that those CNVs with penetrance risk below 25% will not be routinely reported back in the prenatal setting, unless deemed to be clinically actionable. Examples of those situations include; (i) the 15q11.2 microdeletion (*NIPA1* gene), penetrance risk 10.4% (Rosenfeld et al., 2013), associated with developmental delay, behavioural problems, and/or schizophrenia (Doornbos et al., 2009; Sahoo et al., 2011) and which would not be reported; (ii) the 22q11.2 duplication (*TBX1* gene), penetrance risk 21.9% (Rosenfeld et al., 2013), associated with cardiac anomalies. In the absence of cardiac anomalies on initial clinical referral, this would nevertheless be reported and follow-up with detailed ultrasound examination for the presence of cardiac defects would be advised. The main difference between our approach and those which provide all information revealed by array investigation is that the latter will report back; (i) rare/novel inherited CNVs of questionable pathogenicity, and (ii) imbalances for known 'risk loci' but where the future penetrance is uncertain and with low odds.

Information from routine prenatal screening using chromosomal microarrays combined with subsequent long-term follow-up may provide additional valuable information in a cohort with less ascertainment bias than for postnatal diagnosis. In turn, this will help to further determine the full phenotypic spectrum and penetrance risk for certain CNVs, thus allowing for more accurate prenatal counselling in the future. The submission of prenatal CNV findings, along with the detailed prenatal phenotype as well as the subsequent long term outcome and additional postnatal phenotypes (if any) to current databases is needed. This will aid the interpretation process for prenatal chromosomal microarray analysis as has been seen for postnatal use (de Leeuw et al., 2012). Only if rare/novel CNVs identified prenatally, along with the associated phenotypes, are curated into those databases such as DECIPHER (http://decipher.sanger.ac.uk/) and ISCA (https://www.iscaconsortium.org/) can diagnostic

laboratories utilise this valuable information resource, enabling comparison of their findings with those of others thus aiding clinical interpretation. Furthermore, this will likely lead to the detection of novel genes and loci associated with specific foetal abnormalities, as has been successfully observed in the postnatal setting. Nevertheless, the workflow for classification of CNVs, the subsequent interpretation of rare CNVs and risk loci, and the reporting back of results in the prenatal setting warrants further societal research to determine the best provision of care.

The ethical questions posed and the lessons learned in the application of arrays to prenatal diagnosis will assist greatly as sequencing of the foetal exome / genome during pregnancy becomes a possibility. Additionally, the experience currently being gained in research and clinical diagnostic exome / genome sequencing will also assist in considering how these techniques can be best offered in the prenatal setting, perhaps during pregnancy, and ultimately for non-invasive prenatal diagnosis.

7.1.2. Non-Invasive Prenatal Testing (NIPT) for Foetal Aneuploidy Detection

The ability to screen for foetal aneuploidy by non-invasive methods, i.e. by the analysis of cell-free foetal DNA or circulating foetal cells in maternal blood or plasma, has long been considered the 'holy grail' in prenatal genetic diagnosis. The initial work of Lo et al identified circulating cell-free foetal DNA in maternal plasma (Lo et al., 1997). Subsequently, this free foetal DNA was shown to be pregnancy-specific by rapid clearance from the maternal circulation after birth (Lo et al., 1999) and was derived from the trophoblast (Alberry et al., 2007; Faas et al., 2012). Cell-free DNA is fragmented with a size distribution of 160-180bp (Fan et al., 2010), and has been shown to be stable for a period of days post-sampling (Muller et al., 2011). One of the most important factors in maternal blood sampling is the prevention of maternal cell lysis leading to large levels of contaminating cellular DNA (Barrett et al., 2011). Although present at low percentages, from 3% to as much as 20% (Lo et al., 1998; Lun et al., 2008) against a high maternal DNA background, isolation of cell-free DNA has been shown to be feasible for a number of downstream applications of non-invasive prenatal diagnosis (NIPD), including foetal sex assessment, (review and meta-analysis from (Devaney et al., 2011)), and foetal rhesus D blood group genotyping (Finning et al., 2002). NIPD for single gene disorders has thus far been applied for the detection of a broad range of monogenic disorders, but were typically restricted to X-linked disorders or paternally inherited alleles, reviewed by (Bustamante-Aragones et al., 2012).

Advances in detection of foetal cells from maternal circulation have been less successful than for cell-free foetal nucleic acids since foetal cell numbers in maternal circulation are very low and their enrichment and subsequent culture remain challenging (Calabrese et al., 2012; Huang et al., 2011). Clearly, single-cell 'omics' analysis has great potential for NIPD if methods for isolation of foetal cells from the maternal circulation improve. Further research is needed to determine the correlation between individual circulating foetal cells and that of the true foetal genome. Since circulating cell-free DNA is derived from many cells and also represents the entire foetal genome, this may prove to be more representative of the true foetal genome.

Non-invasive foetal aneuploidy detection by massively parallel sequencing was first published by 2 separate groups in 2008, Chiu & Lo et al in Hong Kong (Chiu et al., 2008), and Fan & Quake et al in Stanford (Fan et al., 2008). The massively parallel sequencing approach to aneuploidy detection applies counting statistics to tens or hundreds of millions of sequencing reads in order to identify respective changes in the small percentage of foetal DNA present in the total cell-free DNA sample (Chiu et al., 2009; Fan and Quake, 2010). For example, if 10 million mapped reads are obtained and 1% (100,000 reads) map to chromosome 21 in a normal pregnancy, then a trisomy 21 pregnancy with a level of 10% free-foetal DNA in maternal plasma will contribute a higher proportion of reads to chromosome 21, in this case 1.05% (105,000 reads). With sufficient sequencing coverage (>10 million reads) this small difference can be confidently detected in comparison to the mean and standard deviation (SD) of a normal cohort of pregnancies. In order to distinguish between non-invasive prenatal diagnosis of single gene disorders and the screening for foetal aneuploidy (primarily by massively parallel sequencing), the term non-invasive prenatal testing (NIPT) is now increasingly used.

Since these initial proof-of-principle studies the industry has expanded dramatically, with some tests rapidly reaching clinical use in some countries. In one of the first large-scale clinical validation studies, groups from the UK, the Netherlands and Hong Kong demonstrated detection of trisomy 21 foetuses with 100% sensitivity and 97.9% specificity, which resulted in a positive predictive value of 96.6% and negative predictive value of 100%, using a 2-plex protocol (Chiu et al., 2011). 2-plex implies multiplexing of 2 samples labelled with unique DNA sequence indices clustered within a single lane of a sequencing flow cell. Multiplexing of samples reduces costs per sample, but at the expense of lower sequencing coverage per sample. In comparison, the 8-plex protocol detected 79.1% of the trisomy 21

foetuses and 98.9% specificity, giving a positive predictive value of 91.9% and negative predictive value of 96.9%. Other studies have reported similar high levels of sensitivity and specificity and for additional chromosomes (Palomaki et al., 2012; Ehrich et al., 2011), reviewed in (Verweij et al., 2012). In one of the largest studies to date, Dan et al recently reported on the clinical use of massively parallel sequencing for aneuploidy detection in cell-free DNA from maternal plasma in > 11,000 pregnancies (Dan et al., 2012). 143 cases of trisomy 21 and 47 cases of trisomy 18 were identified correctly, with one false positive case of trisomy 21, one false positive case of trisomy 18 and no false negative cases, indicating 100% sensitivity and 99.96% specificity for the detection of trisomies 21 and 18. Routine clinical use could avoid about 98% of invasive prenatal diagnostic procedures.

Other approaches use targeted strategies for aneuploidy detection of chromosomes 13, 18, 21, X and Y only. Those methods include the analysis of epigenetic differences between foetal and maternal cell-free DNA nucleic acids. One technique uses methylated DNA immunoprecipitation (MeDIP) and real-time quantitative polymerase chain reaction (real-time qPCR) to identify differentially methylated regions (DMRs) between maternal and foetal cell-free DNA (Patsalis et al., 2012; Della et al., 2010; Papageorgiou et al., 2011). This technique was recently shown in a blinded validation study to detect trisomy 21 with 100% sensitivity and 99.2% specificity (Tsaliki et al., 2012).

7.2 Congenital Diaphragmatic Hernia

The exact origins of the early pleuroperitoneal fold (PPF) tissue remain to be resolved, and the mechanisms which are involved in normal PPF development and disrupted in CDH are uncertain. Animal models will remain essential in unravelling these early stages of PPF formation and CDH development due to the difficulties in studying this early stage of foetal development in humans. It has been reported that it is decreased cell proliferation in the PPF which underlies CDH development in the nitrofen rodent model and not increased apoptosis (Clugston et al., 2009). While the nitrofen rodent model has advanced knowledge of diaphragm formation and CDH development, it may not be applicable to understanding the genetic factors involved in human CDH. For this reason, there will likely be a continued shift towards the use of genetic mouse models rather than the nitrofen rodent model to better understand the molecular mechanisms underlying PPF development and the causes of CDH. Expression microarrays have been performed in the *hypoplastic* lungs of the nitrofen rodent as well as in the *hyperplastic* lungs of a normal rodent model following

tracheal ligation (Mesas-Burgos et al., 2010b; Mesas-Burgos et al., 2009). However, global transcriptome analysis has not yet been reported for CDH subsequently treated with tracheal ligation (TL), in the nitrofen rodent or any other model. Recently, the transcriptome of the developing PPFs and muscular diaphragm was investigated in a normal mouse model providing valuable information on which genes are expressed during development of the PPFs and development of the muscular diaphragm in the normal situation (Russell et al., 2012). Of great interest will be future studies in different genetic mouse models of CDH to determine which genes and biological pathways are disrupted in comparison to this normal mouse model dataset. This may help to reveal common developmental network(s) of genes which are dysregulated in specific genetic models of CDH, such as for GATA4, ZFPM2 and NR2F2. Surgical animal models of CDH and lung hypoplasia may also provide a more accurate reflection of response to therapy than with the nitrofen model or genetic models and even identify novel therapeutic targets. We are currently investigating by RNA-Sequencing the effects of tracheal occlusion on gene expression in a surgical rabbit model of CDH, and CDH treated with TO. These results will provide a better understanding of the molecular processes involved in development of lung hypoplasia due to (surgical) CDH and the molecular effects induced by subsequent TO. This will help to identify the biological pathways affected in the hypoplastic lungs due to CDH, as well as the changes induced by TO, thus identifying potential therapeutic targets for further study.

In humans, CDH is considered to have a strong genetic component from classical cytogenetic investigations, reviewed in (Holder et al., 2007). More recently chromosomal microarray analysis of CDH patients has identified novel risk loci and allowed for refinement of other loci (Klaassens et al., 2005; Kantarci et al., 2006; Shaffer et al., 2007; Klaassens et al., 2007; Scott et al., 2007; Wat et al., 2009b; Kantarci et al., 2010; Srisupundit et al., 2010; Rosenfeld et al., 2011; Wat et al., 2011; Yu et al., 2012; Brady et al., 2013a). The two most recent microarray studies of mixed cohorts of isolated and non-isolated CDH patients revealed pathogenic CNVs in 8/45 (2 isolated; 6 non-isolated) and 16/256 (5 isolated; 11 non-isolated) patients investigated, respectively (Wat et al., 2011; Yu et al., 2012). The study of Wat et al (Wat et al., 2011) also reported 16/45 cases with rare inherited CNVs of uncertain clinical significance. Our evaluation into the use of chromosomal microarray analysis for identification of CNVs associated with *isolated* CDH revealed pathogenic submicroscopic CNVs to be a cause in ~9% of foetuses, thus demonstrating the value of chromosomal microarray analysis over conventional karyotyping for gene identification and for investigation of isolated CDH (Brady et al., 2013a). This allowed for refinement of the CDH

locus at 15q26, thus highlighting deletion of NR2F2 as a cause of isolated CDH, and possibly cardiovascular malformations. In addition we add further evidence for recurrent microdeletions at 15q25.2 and 16p11.2 as a cause of CDH, showing apparent high and low penetrance risks of CDH, respectively. Novel loci and rare CNVs have also been identified by us and others revealing new candidate genes and which may be involved in CDH development (Srisupundit et al., 2010; Wat et al., 2011; Yu et al., 2012; Brady et al., 2013a).

While chromosomal microarray analysis has increased the diagnostic yield, revealed novel loci and refined some others, this still does not resolve the genetic cause in the majority of CDH cases. Hence, exome sequencing is an attractive approach for causal gene identification in familial cases of isolated and non-isolated CDH, sequencing all known genes for pathogenic variants responsible for CDH. By applying exome sequencing in a family with 2 prior foetuses affected with isolated CDH we identified a nonsense mutation causing a premature stop codon in the *ZFPM2* gene. The mutation is a cause of isolated CDH in two foetuses, and is also considered the cause of a cardiac defect and diaphragm eventration in a sibling who was shown to carry the same mutation. However, there are also several asymptomatic carriers within this family. These findings add further evidence for heterozygous loss of function mutations (or deletions) which cause haploinsufficiency of *ZFPM2* as a cause of isolated CDH, diaphragm eventration, and possibly cardiovascular malformations. Furthermore, the finding of the same mutation in apparently asymptomatic individuals within the same family demonstrates that *ZFPM2* haploinsufficiency is also associated with reduced penetrance, as well as variable phenotypic expression. Similar findings of incomplete penetrance were recently reported for the *GATA4* gene, for which exome sequencing identified an inherited variant associated with familial CDH, also present in apparently asymptomatic carriers who were later shown by MRI to have mild diaphragm defects (Yu et al., 2013). Taking these findings from exome sequencing and from microarray investigations, it is evident that *NR2F2*, *ZFPM2*, and *GATA4* are all associated with both diaphragm and cardiovascular abnormalities, and that those anomalies can differ in their specific anatomical location and in their severity.

Exome sequencing was also successfully applied to the investigation of syndromic CDH in a second family with 2 foetuses affected with multiple congenital anomalies including microphthalmia, CDH and neural tube defects. A splice site mutation in the X-linked *PORCN* gene was identified, adding further evidence for involvement of *PORCN* and thus Wnt signaling in syndromic forms of CDH in combination with eye anomalies. In a third family

investigated by exome sequencing, a homozygous missense mutation in the *PIGN* gene was identified as the cause of multiple congenital anomalies including CDH in a consanguineous family with a single affected foetus. This finding highlights a possible role for defective GPI anchor synthesis as a cause of syndromic CDH and expands the phenotypic spectrum of developmental abnormalities associated with *PIGN* mutations.

There will undoubtedly be an increase in the use of exome and whole genome sequencing to identify (novel) genetic variants in patients with isolated and non-isolated CDH for research purposes and also in the clinical setting. Targeted mutation analyses on smaller panels of candidate CDH genes in retrospective patient cohorts will also help determine the prevalence in sporadic cases of isolated CDH. By firstly screening for CNVs, followed by exome or whole genome sequencing in large cohorts of isolated CDH patients it becomes possible to search for an excess burden of rare variants in candidate CDH genes. This technique has been applied to autism and to congenital heart disease (O'Roak et al., 2012; Zaidi et al., 2013). To aid variant identification, the use of functional predictions and gene/variant prioritizations will play an increasing role (Sifrim et al., 2013).

Exome sequencing is rapidly entering clinical use; however, it does have its limitations. The capture methodology has advanced from 'on array' to 'in solution' capture kits, and the target designs continue to improve in an attempt to provide more even coverage of those target regions. An example of the limitations of exome sequencing is a SMOC2 mutation causing major dental developmental defects which was missed by whole-exome sequencing, but was identified by homozygosity mapping of affected individuals and candidate gene prioritization (Bloch-Zupan et al., 2011). Chromosomal microarrays are now in mainstream diagnostic use for postnatal use and rapidly entering the prenatal setting. Given the costs of exome sequencing, an array is almost always performed beforehand to exclude pathogenic CNVs as the cause. Copy number analysis has been demonstrated using exome sequencing data (Krumm et al., 2012), but the reliability for diagnostic purposes remains uncertain, and continued changes to capture design can complicate or preclude routine copy number detection. There is therefore a 'blind spot' in the search for pathogenic variants between that offered by array (typically several kilobases to several hundred kilobases in size) to the single nucleotide variants and indels which are able to be detected by exome sequencing. Without the full picture, identifiable pathogenic variants in this range still go undetected.

Numerous genes and genomic loci are associated with an increased risk of CDH, but none of those display full penetrance for CDH. As our knowledge of the genetic factors influencing

CDH improves, identification of the genetic factors which influence CDH penetrance will become increasingly important and may reveal novel therapeutic targets. The low familial recurrence risk for cases of isolated CDH (1-2%) points towards complex polygenic and/or epigenetic factors playing an important role. Future studies may be undertaken to identify 2nd genetic hits which may contribute to CDH penetrance in CDH patients with a primary pathogenic genetic variant detected. Girirajan et al proposed a 'two-hit model' for severe developmental delay in association with the recurrent 16p12.1 microdeletion (Girirajan et al., 2010). The same authors later demonstrated that among children with intellectual disability or congenital anomalies, 10.1% carried a second large copy-number variant in addition to the primary genetic lesion (Girirajan et al., 2012). Our finding of a 17p12 duplication (CMT1A locus; OMIM #118220) in a foetus with a 15q25.2 deletion and CDH may represent a 'second CNV hit' contributing to CDH penetrance or severity. Can rare variants be identified which represent a 2nd genetic hit, either de novo or inherited? Is it possible to identify candidate modifier genes influencing CDH penetrance in animal models, for example, the *ZFPM2*, *NR2F2*, and *GATA4* mouse models? The *FREM1* gene was recently associated with CDH in humans and in the Frem1 (eyes2) mouse model (Beck et al., 2013b). The authors subsequently showed in an alternative Frem1 mouse model that variants affecting GATA4 and SLIT3 expression modulated certain phenotypic features (Beck et al., 2013a).

In addition to the whole genome sequence of an individual, the ongoing monitoring of other '-omes' (e.g. transcriptome, methylome, proteome, metabolome) from the same individual has been termed the integrated personal omics profile (or iPOP), and a proof of principle has been demonstrated (Chen et al., 2012). This type of information combing the genetic background along with ongoing dynamic monitoring an individual's physiological state may eventually lead to truly personalised medicine. This integrated approach has the potential to improve assessment of an individual's risk of disease, detect disease onset and monitor disease progression, aid with targeted treatments, improve our understanding of the biological processes underlying disease states and the variation in disease severity and response to treatment between individuals (Chen et al., 2012; Li-Pook-Than and Snyder, 2013). These types of techniques will increasingly be applied to the study of congenital malformations such as CDH.

By applying RNA sequencing we sought to explore the gene expression profiles in the amniotic fluid cells in a cohort of isolated CDH foetuses. This analysis identified 2 groups

representing potential molecular subtypes of CDH, each group comprising ~1/4 of the total studied. In one group of CDH foetuses downregulation of TGFB1 and the upregulation of TNF are predicted to cause much of the downstream dysregulation in gene expression observed and may thus represent therapeutic targets. This subtype also shows downregulation of *CTGF*, likely as a direct result of TGFB1 downregulation. CTGF has previously been shown to be downregulated in the nitrofen rodent model of CDH and lung hypoplasia (Mesas-Burgos et al., 2010a; Mesas-Burgos et al., 2010b; Mesas-Burgos et al., 2009). Interestingly, tracheal ligation was shown to upregulate *CTGF* in the rodent model (Mesas-Burgos et al., 2010a; Mesas-Burgos et al., 2009). In contrast to some of the targeted studies of human CDH, we did not observe significant dysregulation of key genes involved in retinol metabolism. Tracheal fluid can be retrieved from CDH patients undergoing FETO prior to tracheal occlusion and following balloon removal. This may represent a valuable source of material which could be studied to better understand the differences in response to foetal therapy.

The investigation of environmental factors is challenging in human subjects, however, there is some evidence which supports possible defects in foetal retinol metabolism (Beurskens et al., 2009). Beurskens et al reported lower levels of retinol and RBP in cord blood of newborns with CDH compared to controls, independent of maternal levels (Beurskens et al., 2010). The same authors also reported an association between reduced dietary Vitamin A intake and an increased of CDH (Beurskens et al., 2013). Targeted gene expression studies in foetal skin fibroblasts have demonstrated expression changes in genes involved in retinol metabolism in a small number of CDH patients (Goumy et al., 2010a). These studies are few and on small cohorts, therefore these results need to be reproduced in larger populations to determine the significance of defects in foetal retinol metabolism.

Novel cellular models are likely to play an increasing role in the study and treatment of CDH. It has been demonstrated that mesenchymal stem cells (MSCs) can be routinely isolated from the amniotic fluid (Zia et al., 2013). These AF-MSCs provide a source of cells from the patient which can be studied and manipulated (Zia et al., 2013). The availability of AF-MSCs represents a source of the patients own material which could potentially be used to engineer an autologous tissue patch during the pregnancy which is ready for repair of the diaphragm after birth (Turner et al., 2011). A successful stem-cell-based tissue engineered tracheal replacement was recently performed in a child with congenital tracheal stenosis (Elliott et al., 2012). The potential for tissue engineering an autologous lung by seeding a

decellularised extracellular matrix (ECM) scaffold has also been demonstrated using a refined protocol which retained the pulmonary vascular and bronchial microarchitecture (Maghsoudlou et al., 2013). The potential for AF stem cells to rescue lung growth and function both *in vivo* and *in vitro* in the nitrofen rodent model of CDH and lung hypoplasia has also been shown (Pederiva et al., 2012).

Over the past decade there has been a steady improvement in our understanding of the genetic factors contributing to CDH by the application of chromosomal microarrays and more recently exome sequencing. Chromosomal microarrays are increasingly being used in the prenatal setting and it is likely that exome sequencing will be increasingly used for diagnostic purposes including prenatal diagnosis. The treatment for CDH patients has also advanced and shifted to the prenatal setting in an attempt to improve lung growth and function prior to birth. Advances in tissue engineering and the availability of foetal stem cells by amniocentesis sampling during pregnancy show great promise for improving the outcome and treatment options available. The use of next generation sequencing technologies for exome / whole genome analysis and transcriptome studies in CDH patients and in animal models will continue to develop our understanding of the condition and the effects of available treatments, allowing for prediction of which patients will respond best to therapy and understanding why, thus leading to novel therapeutic treatments which in turn will reduce the high mortality rate and morbidity associated with congenital diaphragmatic hernia.

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Professional Profile

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Participation at international meetings

Belgian Society of Human Genetics (BeSHG) 14th Annual Meeting.	Antwerp, Belgium	February 7, 2014	Exome Sequencing Identifies a recessive PIGN splice site mutation as a cause of Syndromic Congenital Diaphragmatic Hernia	Poster
Congenital Diaphragmatic Hernia Conference & Workshop 2013	Rotterdam, Netherlands	9-11 June, 2013	Exome sequencing identifies inherited pathogenic variants for congenital diaphragmatic hernia	Oral Presentation
Genomic Disorders 2013: From 60 years of DNA to human genomes in the clinic.	Homerton College, Cambridge University, Cambridge, UK.	10-12 April, 2013	Exome Sequencing Identifies Inherited Pathogenic Variants for Congenital Diaphragmatic Hernia	Poster (P25)
Belgian Society of Human Genetics (BeSHG) 13th Annual Meeting.	EXPO, Brussels, Belgium	March 15, 2013	Exome Sequencing Identifies Inherited Pathogenic Variants for Congenital Diaphragmatic Hernia	Poster (56)

European Human Genetics Conference (ESHG) 2012	Nürnberg, Germany	June 23 - 26, 2012	Prenatal array CGH identifies genomic imbalances associated with congenital diaphragmatic hernia (CDH)	Poster
Congenital Diaphragmatic Hernia: More Questions than Answers?	Rome, Italy.	1-3 February 2011	Array CGH Identifies Novel Recurrent & Non-Recurrent Genomic Imbalances associated with CDH	Oral Presentation
2nd Genomics & Proteomics Workshop 2011	Macedonia Academy of Science & Arts, Skopje, Macedonia.	27-29 June, 2011	Genomic Arrays in Diagnostics	Invited speaker and workshop leader
European Human Genetics Conference (ESHG) 2011	Amsterdam, Netherlands	28-31 May, 2011	The Use of Array CGH for Prenatal Diagnosis of Fetuses with Congenital Malformations detected by Ultrasound	Oral Presentation Nomination Young Investigator Award
Belgian Society of Human Genetics (BeSHG) 12th Annual Meeting "Next Generation Sequencing and Recent Advances in Genetics"	Liège, Belgium	March 2, 2012	Prenatal Array CGH Identifies Genomic Imbalances Associated With Congenital Diaphragmatic Hernia (CDH)	Poster (101).
International Society for Prenatal Diagnosis (ISPD) 15th International Conference on Prenatal Diagnosis and Therapy	Amsterdam, Netherlands	11-14 July 2010	Array CGH for the Identification of Genomic Imbalances Associated With Congenital Diaphragmatic Hernia & Prenatal Congenital Anomalies	Poster
Belgian Society of Human Genetics (BeSHG) 10th Annual Meeting: "The Dark Side of the Human Genome"	Ghent, Belgium, February 26, 2010 (42).	February 26, 2010	The use of array CGH for the detection of genomic imbalances in patients with congenital diaphragmatic hernia	Poster (42)
CDH Euro-Consortium – Advances in Congenital Diaphragmatic Hernia	Mannheim, Germany	29-30 October 2009	The use of array comparative genomic hybridisation (aCGH) for the detection of genomic imbalances in patients with CDH	Oral Presentation
European Human Genetics Conference (ESHG) 2009	Vienna, Austria.	May 23-26, 2009	Comparison of 1 Mb BAC array and 105K oligo array in a clinical diagnostic setting	Poster Presentation

Courses & Workshops.

NGS Course: Medical Genomics. 17-20 September 2013, Leuven, Belgium.

FTLNS Workshop: The Epigenetics Revolution – development & disease biology revisited. 1-4 July 2013, Leuven, Belgium.

Belgian Medical Genomics Initiative. 18 June 2013. Leuven, Belgium.

FTNLS Workshop: Next Generation Sequencing. 17-21 September 2012. Leuven, Belgium.

5th Benelux NGS Conference. 20 June, 2012. Leuven, Belgium.

2nd Genomics & Proteomics Workshop. 27-29 June, 2011. Skopje, Macedonia.

Arrays in Daily Practice - Promises & Pitfalls. One day Symposium. May 27, 2011. Amsterdam, Netherlands.

Post-Graduate Course: Congenital Diaphragmatic Hernia. 01 February, 2011. Bambino Gesu Childrens Hospital, Rome Italy.

Advances in Genomics Symposium. 28-29 January, 2010. Ghent, Belgium.

Molecular Karyotyping Workshop. 21-22 January, 2010. Leuven, Belgium.

1st Goldrain Course in Prenatal Genetic Diagnosis. 19-24 September, 2009. Goldrain, South Tyrol, Italy.

Permanent Education Course in Human Genetics / Inter-University Certificate in Human Genetics. 2008/2009. Belgium.

Introduction to R: Statistical Computer Programming. 23-26 February, 2009. Leuven, Belgium.

Array Workshop. 2008. Leuven, Belgium.

Supervision of KU Leuven Masters Students;

Ariane Tyberghein. "The detection of mosaicism in prenatal samples using array CGH technology."

Karolien Bijnens. "SUFU is a candidate gene for holoprosencephaly in a prenatal patient with a de novo deletion of 10q24."

Ann Sophie Walravens. "Congenital Diaphragmatic Hernia: Array CGH for the Detection of Aberrations Causal For CDH."

Radost Ratcheva. "Use of custom design array CGH for CDH patients."

Nathalie Fieremans. "Validation of the 180K oligo arrays for the detection of chromosomal imbalances in humans."

Popular Summary

This doctoral research has focused on the application of recently emerging genetic technologies to prenatal genetic diagnosis. Conventional cytogenetic analysis by karyotype visualizes stained chromosomes by light microscopy. Karyotyping can detect numerical (e.g. trisomy 21, Down syndrome) and structural rearrangements to a resolution of ~10Mb. Furthermore, karyotyping requires the culturing of foetal cells obtained by invasive procedures which requires at least 8-10 days.

Chromosomal microarray analysis is a recent technique which can detect Copy Number Variations (CNVs), i.e. gains or losses of regions of the genome, at a far superior resolution and in a faster timeframe compared to conventional karyotyping. This makes chromosomal microarrays an attractive alternative to karyotyping for prenatal diagnosis. The technology has entered mainstream clinical use over the past several years primarily for the genetic diagnosis of children with multiple congenital abnormalities, as well as isolated abnormalities such as congenital heart defects, children with developmental problems, and autism. Prenatal diagnosis has been left lagging behind somewhat. One of the major reasons for the slow introduction into prenatal diagnosis has been due to the detection of CNVs which, based upon our current knowledge, are of uncertain clinical significance. These variants of uncertain significance (VOUS) are observed in 1-2% of patients and normal individuals studied. Such findings during pregnancy can lead to increased anxiety for the parents and are difficult to counsel for. Furthermore, CNVs may be detected which are associated with reduced penetrance (i.e. they have been observed in both normal individuals and in patients; for example, an affected child has inherited the same CNV from an apparently normal asymptomatic parent). Alternatively, a CNV may be associated with variable expression for which a spectrum of phenotypic features have been observed in patients. This creates particular challenges in the prenatal setting, since it is not possible to predict the phenotypic features, nor their severity, in the future child. It should also be mentioned that all of us carry benign polymorphic CNVs, some of which may be common within particular populations, and some which are rare and only observed in individual families. With increasing resolutions, the boundary between pathogenic CNVs and benign CNVs becomes blurred creating challenges for the interpretation and counseling of families.

We therefore sought to investigate the clinical utility of prenatal genetic diagnosis by chromosomal microarray analysis in those pregnancies where foetal abnormalities were detected on ultrasound. Furthermore, we also considered how to handle the reporting of different types of CNVs during pregnancy. Our results demonstrated an increase in the

diagnostic yield of pathogenic variants compared to conventional karyotyping alone; in ~3% of cases a submicroscopic pathogenic CNV was detected which would have gone undetected by conventional karyotyping; in a further ~4% of cases with an abnormal karyotype but for which the chromosomes involved could not be fully elucidated, chromosomal microarray analysis provided valuable information aiding the interpretation; in ~3% of cases the genetic abnormality detected was also detected by karyotype. These advantages however come with the detection of VOUS in 1-2% of foetuses (and parents) studied.

Clearly pathogenic CNVs are always reported back to the parents, whereas common polymorphic CNVs are not routinely reported back in the diagnostic setting (both in the post- and prenatal setting). In contrast to postnatal reporting criteria, we have taken the approach not to report VOUS back to parents during the pregnancy, since this is of limited (or no) clinical utility and may even cause more anxiety to the parents. For those CNVs which pose a penetrance risk >25 %, these are reported and counseled for based upon the evidence from large case-control studies; those with a penetrance risk of <25%are not (routinely) reported to the parents unless actionable.

Congenital Diaphragmatic Hernia (CDH) is an example of a foetal abnormality detectable by ultrasound during pregnancy. The abnormal development and closure of the diaphragm during foetal development allows herniation of abdominal organs including the intestines, and often the liver, into the thoracic cavity. This causes reduced space for the lungs to develop and grow resulting in hypoplastic lungs which are then underdeveloped at birth causing great difficulties for the newborn to breathe. In association, the vasculature of the lungs is also abnormal causing pulmonary hypertension. Until the newborn is stable it is not possible to surgically correct the diaphragm defect, and mortality remains high in the most severe cases. UZ Leuven is a specialist referral centre for pregnant women with foetuses affected with isolated CDH. In those with moderate-severe lung hypoplasia, a minimally invasive foetal therapy is offered by fetoscopic endoluminal tracheal occlusion (FETO). This involves the placement of an inflatable balloon into the trachea of the foetus in utero. By 'plugging' the trachea, there is an increase in pressure within the lungs due to the natural secretion of lung fluids which are unable to escape. This causes inflation of the lungs counteracting the effects of the herniated organs, and thus improves lung growth during pregnancy while the foetus does not need to breathe, thereby improving the outcome for the newborn and reducing the neonatal mortality to some degree.

We have focused on identifying genetic factors which contribute to CDH pathogenesis in order to improve our understanding of the underlying genes and molecular mechanisms

involved. By chromosomal microarray analysis we demonstrated that submicroscopic CNVs associated with CDH were detected in at least 10% of foetuses studied. These genomic variants would have gone undetected by conventional karyotyping. This is significantly higher than the increase in detection rate we observed in pregnancies with any ultrasound abnormality, as discussed above. Thus, isolated CDH represents a valid reason for performing chromosomal microarray analysis and suggests that for these patients the utility of conventional karyotyping is of limited use.

However, for the majority of patients still no genetic diagnosis can be obtained. In order to further identify the genetic variants responsible, we applied next-generation sequencing (NGS) technologies in order to sequence the exome (all the genes in the human genome) in familial cases of isolated CDH, and non-isolated or syndromic CDH. We demonstrated that in several families with a high likelihood of a genetic cause due to multiple affected foetuses, or due to a consanguineous relationship in the parents, this approach was able to identify the pathogenic mutation. This provides identification of the cause and allows for a more accurate calculation of the recurrence risk for future pregnancies in these families, as well for other family members where appropriate.

In conclusion, we have demonstrated that the use of chromosomal microarray analysis for prenatal diagnosis in the presence of ultrasound abnormalities increases the detection of genetic variants which explain the foetal abnormalities. This provides families with an explanation for this occurrence and thus a more accurate assessment of the recurrence risk for future pregnancies. We demonstrated that isolated CDH is particularly suited to chromosomal microarray analysis, by identification of novel CNVs, as well as confirming the association of other CNVs with CDH. Furthermore, we show that where chromosomal microarray analysis does not reveal a cause that the use of NGS technologies for exome sequencing can provide a diagnosis for some families. Chromosomal microarray analysis is now offered as the first-tier test for prenatal diagnosis in Leuven, and we anticipate that exome sequencing will increase in use as costs of NGS continue to fall. However, caution must be maintained and the ethical challenges posed by these new techniques need to be carefully addressed in order to ensure that the benefits outweigh the concerns of potentially identifying ‘unsought for findings’ or ‘secondary variants’ which are unrelated to the initial reasons for investigation.

Samenvatting

Prenatale genetische diagnostiek gebeurt traditioneel door middel van karyotypering. Foetale cellen worden verkregen van het amniosvocht of van chorionvlokken via een invasieve staalafname. Deze cellen worden vervolgens opgekweekt en de metafase chromosomen worden gevisualiseerd door kleuring. Karyotypering laat toe om numerieke afwijkingen (b.v. het syndroom van Down of trisomie 21) en structurele afwijkingen met een resolutie van ~10Mb te detecteren. Dit duurt ongeveer 8 à 10 dagen.

Chromosomale microarray analyse is een recente techniek die toelaat deleties en duplicaties in het genoom, ook copy number variations (CNV's) genoemd, sneller op te sporen en dit met een veel hogere resolutie in vergelijking met conventionele karyotypering. Hierdoor zijn chromosomale microarrays een heel interessant alternatief. De technologie werd de laatste jaren klinisch ingevoerd voor de genetische diagnostiek van kinderen met ontwikkelingsstoornissen zoals hartafwijkingen, intellectuele beperking en autisme. De implementatie van microarrays in de prenatale diagnostiek verloopt echter moeizamer. Een belangrijke reden voor de moeizamere implementatie is dat voor een groot deel van de CNV's met de huidige kennis de klinische betekenis nog onduidelijk is en dat het tot nog toe onmogelijk is om te voorspellen hoe deze CNV's het toekomstige fenotype zullen beïnvloeden.

Een eerste doel van deze thesis was de ontwikkeling en introductie van chromosomale microarrays in de klinische diagnostiek. Wij onderzochten het klinische voordeel van microarrays in de prenatale genetische diagnose van zwangerschappen waarbij foetale abnormaliteiten gedetecteerd werden via echografie. Daarnaast werd ook een methodiek ontwikkeld om welbepaalde CNV's te rapporteren. Onze studie toonde een belangrijk diagnostisch voordeel in vergelijking met conventionele karyotypering. In 3% van de gevallen werden submicroscopische pathogene CNV's gedetecteerd die niet opgepikt werden met conventionele karypotypering. In 4% van de foetussen werd een abnormaal karyotype gedetecteerd, maar leverde de microarray analyse waardevolle bijkomende informatie.

Pathogene CNV's worden altijd gerapporteerd aan de ouders, daar waar goedaardige CNVs binnen een diagnostische context (zowel post- als prenataal) niet worden gerapporteerd. In tegenstelling tot de rapporteringsmethodiek in de postnatale diagnostiek, hebben we er voor gekozen om ouders tijdens een zwangerschap niet te informeren over CNV's met ongekende gevolgen, gezien ze weinig of geen informatie bevatten en bovendien kunnen leiden tot een grotere ongerustheid bij de ouders. Een van de foetale afwijkingen die

echografisch zichtbaar is, is congenitale diafragmatische hernia (CDH). De abnormale ontwikkeling en sluiting van het diafragma (middenrif) tijdens de ontwikkeling van de foetus zorgt ervoor dat verschillende organen, waaronder de ingewanden en de lever, zich kunnen verplaatsen naar de borstholte. Dit beperkt de ruimte voor de groei van de longen waardoor deze onderontwikkeld zijn bij de geboorte. Hierdoor worden ernstige ademhalingsproblemen veroorzaakt bij de pasgeborene. Bovendien is ook het bloedvatenstelsel abnormaal wat pulmonale hypertensie veroorzaakt. Totdat de zuigeling stabiel is kan een dergelijk defect in het diafragma niet hersteld worden. Hierdoor is de mortaliteit groot bij de sterkst aangetaste patiënten. Het UZ Leuven heeft een gespecialiseerd centrum waarnaar de meeste vrouwen die zwanger zijn van een foetus met een geïsoleerde CDH doorverwezen worden. Voor de foetussen die lijden aan een matige tot zware onderontwikkeling van de longen (longhypoplasie) wordt een zogenaamde Foetoscopische Endoluminele Tracheale Occlusie (FETO) aangeboden. Bij deze kleine chirurgische ingreep wordt in utero een kleine ballon aangebracht in de luchtpijp van de foetus. De luchtpijp wordt op die manier geblokkeerd waardoor het door de longen geproduceerde vocht vastgehouden wordt en de druk in de longen stijgt. Hierdoor gaan de longen wat opblazen wat de druk afkomstig van de migrerende organen tegenwerkt. Bovendien wordt de longgroei gestimuleerd terwijl de foetus niet hoeft te ademen, wat de impact van CDH in de pasgeborene vermindert en de neonatale mortaliteit gedeeltelijk reduceert.

Hoewel er in modelorganismen verschillende genen werden geïdentificeerd die CDH kunnen veroorzaken, waren er voor de start van deze thesis nog maar weinig genetische oorzaken geïdentificeerd. Deze thesis stelde zich als doel om de genetische factoren die bijdragen aan CDH te identificeren. Met behulp van chromosomale microarray analyse detecteerden we submicroscopische CNV's geassocieerd met CDH in 10% van de bestudeerde foetussen. Deze genomische varianten konden tot nu toe niet gedetecteerd worden met conventionele karyotypering. Deze analyse liet toe om een aantal genen te identificeren die bij een teveel of een tekort aanleiding kunnen geven tot CDH. Het diagnostische voordeel in deze groep is significant hoger dan het voordeel in de groep van zwangerschappen met een abnormale echografie (zie hoger). De echografische detectie van CDH in een foetus is bijgevolg een indicatie om chromosomale microarray analyse uit te voeren. Hoewel CNV's een belangrijke oorzaak zijn van CDH, kan voor de meerderheid van de patiënten de genetische oorzaak (nog) niet worden geïdentificeerd. Recent werd het mogelijk om aan een redelijke kost de sequentie te bepalen van het volledige genoom (of alle exonen) van een individu. Om

mogelijke nieuwe genen te identificeren die betrokken zijn in de ontwikkeling van het diafragma, hebben we via next-generation sequencing (NGS) het exoom (al de genen van het humane genoom) in families met geïsoleerde of syndromische CDH geanalyseerd. Deze aanpak liet toe om een aantal pathogene mutaties te identificeren.

Samenvattend kunnen we stellen dat chromosomale microarrays bij een abnormale echografie de detectie van oorzakelijke genetische mutaties verhoogt. Hierdoor kan de genetische oorzaak gerapporteerd worden aan de betrokken families en kan het risico voor volgende zwangerschappen berekend worden. Het gebruik van deze test liet ons toe om enerzijds nieuwe CNV's oorzakelijk voor CDH te identificeren en anderzijds de associatie van welbepaalde CNV's met CDH te bevestigen. Indien er geen pathogene CNV's gedetecteerd werden met behulp van chromosomale microarray analyse, stelden we vast dat sequentiebepaling van het exoom toch toelaat om oorzakelijke mutaties te identificeren. Chromosomale microarray analyse is nu de test die bij voorkeur wordt aangeboden voor prenatale diagnose in het UZ Leuven, en we voorspellen dat het gebruik van exoom sequentiebepaling zal stijgen met de steeds dalende prijzen. Wel dient het gebruik van deze nieuwe technologieën met enige voorzichtigheid te gebeuren en dienen de ethische aspecten ervan goed overwogen te worden.

Scientific Summary

Chromosomal microarray analysis has gradually replaced conventional karyotyping over recent years in the postnatal setting which has revolutionized whole genome screening for genomic imbalances in patients. We sought to evaluate the benefits and the challenges of applying chromosomal microarrays to prenatal diagnosis for referrals with abnormal ultrasound findings. Our findings, presented in Chapter 3, demonstrate a diagnostic yield of ~10%. Importantly, ~3% are caused by submicroscopic CNVs which would go undetected by conventional karyotyping alone. Furthermore, the higher resolution offered by chromosomal microarray analysis led to important additional information in ~4% of patients. Of particular interest we discover a novel and unexpected advantage of arrays; a 500kb paternal insertional translocation is the likely driver of a de novo unbalanced translocation, thus improving recurrence risk calculation in this family. Our study has, in part, paved the way for the recent 'Summary Guidelines for Prenatal Chromosomal Microarray Analysis and Genetic Counselling' from the Belgian Society for Human Genetics [http://www.beshg.be/download/annex_2_summary_of_array_and_prenatal_guidelines_20130502.pdf]. The implementation of prenatal chromosomal microarray analysis as the first tier test in place of conventional karyotyping brings the standard of prenatal diagnosis in line with that which is provided for postnatal genetic diagnosis.

Congenital diaphragmatic hernia (CDH) is a life-threatening prenatal disorder detectable by ultrasound during pregnancy. We sought to further unravel the genetic factors underlying isolated CDH by the design of a custom microarray covering genomic loci recurrently associated with CDH and candidate CDH genes. Our retrospective screen of 79 isolated CDH patients using this custom microarray is presented in Chapter 4. This study identified a novel duplication of the EFNB1 gene in a male patient which was considered likely to be pathogenic. Since our publication, a second case of a male CDH patient with duplication of EFNB1 was reported, thus reinforcing EFNB1 dosage sensitivity as a cause of isolated CDH. In order to further identify (novel) CNVs and genes associated with isolated CDH, we undertook a prospective prenatal study using chromosomal microarrays with genome-wide coverage in 75 foetuses with isolated CDH, which is presented in Chapter 4. This study revealed submicroscopic de novo pathogenic CNVs in 9.3% and rare inherited variants which may be involved in CDH in a further 4% of foetuses. This diagnostic yield is significantly higher than the ~3% rate of pathogenic submicroscopic CNVs which we observed in our prenatal study using the same microarray platform. Isolated CDH thus represents a valid cohort for CNV screening in the prenatal phase, and suggests that the clinical utility of

conventional karyotyping is questionable for this group of patients. This study allowed us to further refine the critical region at 15q26 to only 2 genes, pinpointing NR2F2 as the causal gene. We add further evidence for the 15q25.2 and 16p11.2 recurrent microdeletions as CDH loci, and we identify novel CNVs not previously observed in association with CDH, including a duplication of 4p15.2-p14.

We next evaluated the use of exome sequencing for the investigation of isolated CDH and non-isolated CDH where a genetic cause was suspected. Our results show that exome sequencing represents an effective technique with which to investigate familial CDH, described in Chapter 5. In the first family studied, we identified a nonsense mutation in ZFPM2 in 2 individuals with isolated CDH, as well as a sibling with a congenital heart defect. Surprisingly, the mutation was shown to be transmitted from the unaffected mother, and is also carried by the maternal grandfather and the maternal sister, both of whom are also asymptomatic. This intriguing finding highlights the complexity of CDH, reinforcing the involvement of additional as yet unidentified (epi)genetic factors in CDH penetrance. In a second family with 2 male foetuses with MCA, we identify a mutation in the X-linked PORCN gene inherited from an unaffected mother who was shown to have extreme skewing of X-inactivation. This further implicates Wnt signaling as playing a role in CDH, as well as multiple aspects of foetal development. In a third consanguineous family we identify a mutation in PIGN in a foetus with MCA, inherited from carrier parents. PIGN is involved in GPI anchor synthesis and our finding adds to a growing body of evidence that defective GPI anchor synthesis causes multiple phenotypes in humans.

Given the variation in severity of herniation and thus pulmonary hypoplasia, as well as differences in responses to foetal and / or neonatal therapy for CDH patients, we sought to explore whether gene expression analysis of amniotic fluid cells from CDH foetuses could identify dysregulated genes and biological pathways which may act as predictive biomarkers. In this exploratory study we applied RNA-sequencing to investigate gene expression in cultured cells sourced from amniotic fluid of isolated CDH patients, described in Chapter 6. This analysis identifies 2 potential molecular subtypes of isolated CDH, one of which is characterized by downregulation of TGFB1 and CTGF, and upregulation of TNF, IL6 and IL8. This highlights downregulation of TGFB signalling as a likely cause of much of of the downstream dysregulation in gene expression observed, including that of CTGF which has been previously implicated in the nitrofen rodent model of CDH. Furthermore, this group of patients shows an apparent inflammatory response indicated by the upregulation of TNF, IL6 and IL8 which may in turn exacerbate postnatal pulmonary hypertension. These findings

direct future targeted studies in a larger cohort of isolated CDH patients to determine the clinical significance and therapeutic potential.

Wetenschappelijke samenvatting

Chromosomale microarray analyse heeft de laatste jaren conventionele karyotypering geleidelijk aan vervangen als diagnostische test bij patiënten met ontwikkelingsstoornissen. Deze evolutie is het gevolg van de hogere resolutie van chromosomale microarray analyse waardoor kleinere oorzakelijke kopij variaties kunnen worden gedetecteerd. In deze thesis evalueerden we de voordelen evenals de verschillende uitdagingen betrokken bij de implementatie van chromosomale microarray analyse in de prenatale diagnostiek, vooral bij foetussen waar er een echografische afwijking werd gedetecteerd. Onze resultaten, beschreven in hoofdstuk 3, tonen bij 10% van de foetussen een pathogene kopij variatie (copy number variation of CNV). Hierbij wordt 3% veroorzaakt door submicroscopische CNV's die niet gedetecteerd zouden worden met conventionele karyotypering. Bovendien zorgde de hogere resolutie van chromosomale microarray analyse voor belangrijke extra informatie bij ~4% van de patiënten. Daarnaast werd ook een nieuw en onverwacht voordeel van arrays ontdekt: de identificatie van een paternele insertionele translocatie die naar alle waarschijnlijkheid de oorzaak was van een de novo ongebalanceerde translocatie bij het kind. De mogelijkheid om dergelijke fouten te identificeren bij ouders, laat toe het risico voor volgende zwangerschappen accurater in te schatten. Onze studie heeft gedeeltelijk de krijtlijnen uitgezet voor de recente 'Summary Guidelines for Prenatal Chromosomal Microarray Analysis and Genetic Counselling' van de Belgian Society for Human Genetics [http://www.beshg.be/download/annex_2_summary_of_array_and_prenatal_guidelines_20130502.pdf].

Congenitale hernia diafragmatica (CDH) is een levensbedreigende prenatale aandoening die gedetecteerd kan worden via echografie. Hoewel er reeds veel informatie over de ontstaansmechanismen van CDH gekend is door de studie van modelorganismen, blijft de kennis over de genetische basis van deze aandoening bij de mens fragmentarisch. Gezien men op basis van de genetische afwijking een prognose kan maken over het ontwikkelingspotentieel van de foetus, zou dergelijke kennis kunnen helpen bij het beslissingsproces over het al dan niet overgaan tot foetale therapie. Daarnaast zou een betere kennis van de pathogenese van CDH kunnen leiden tot een betere foetale therapie.

Ons doel was om de genetische factoren die geïsoleerde CDH veroorzaken verder te ontrafelen. Hiervoor ontwikkelden we een microarray die toeliet om kopij variaties te identificeren in loci en kandidaatgenen die eerder geïdentificeerd werden via klinische studies en studies in modelorganismen. Met behulp van deze microarray voerden we een

retrospectieve studie uit op 79 patiënten met geïsoleerde CDH, waarvan de resultaten zijn weergegeven in hoofdstuk 4. In deze studie rapporteren we een mannelijke patiënt met een duplicatie in EFNB1 als mogelijk nieuwe genetische oorzaak van CDH. Na onze publicatie werd ook in een tweede mannelijke patiënt een duplicatie van het EFNB1 gen vastgesteld, wat de evidentie versterkt dat gevoeligheid voor duplicaties en deleties van EFNB1 een oorzaak is van geïsoleerde CDH. Om vervolgens meer CNV's en genen betrokken bij CDH te identificeren startten we ook een prospectieve prenatale studie in 75 foetussen met geïsoleerde CDH. De resultaten van deze studie, waarvoor we eveneens genoomwijde chromosomale microarrays gebruikten, worden ook beschreven in hoofdstuk 4. Deze studie ontdekte de novo submicroscopische pathogene CNV's in 9.3% van de foetussen en zeldzame, overgeërfde varianten die mogelijks betrokken zijn bij CDH in 4% van de foetussen. Foetussen met geïsoleerde CDH vormen dus een waardevolle groep voor CNV screening tijdens de prenatale fase. Ons onderzoek toont bovendien aan dat voor deze groep het nut van conventionele karyotypering beperkt is. Deze studie liet ook toe om de kritische gedeleteerde regio op 15q26 te verfijnen tot 2 genen, waarbij NR2F2 als het oorzakelijke gen wordt aangeduid. We brengen ook extra evidentie aan dat 15q25.2 en 16p11.2 recurrente microdeleties oorzakelijk zijn voor CDH. We identificeerden bovendien nieuwe CNV's, waaronder een duplicatie van 4p15.2-p14, die nog niet eerder werden geassocieerd met CDH.

Vervolgens evalueerden we mogelijkheid om de oorzaak van geïsoleerde en niet-geïsoleerde CDH te bepalen via exonische sequentiebepaling. Onze resultaten tonen aan dat exon sequencing een efficiënte techniek is om familiale CDH te onderzoeken, zoals beschreven in hoofdstuk 5. In een eerste familie werd een nonsense mutatie in ZFPM2 gedetecteerd in 2 foetussen met geïsoleerde CDH, en in hun broer met een aangeboren hartafwijking. De mutatie werd onverwacht doorgegeven via de niet aangetaste moeder, en komt ook voor bij de maternele grootvader en de maternele tante die beiden asymptomatisch zijn. Deze resultaten wijzen op variabele penetrantie van de mutatie en tonen de moeilijkheid aan om mutaties oorzakelijk voor CDH te identificeren. In een tweede familie met 2 mannelijke foetussen met multipele afwijkingen werd een mutatie in het X-gebonden PORCN gen geïdentificeerd. De mutatie werd overgeërfd van de asymptomatische moeder waarbij 'skewed' X-inactivatie werd vastgesteld. Gezien PORCN betrokken is in het intracellulaire transport van Wnt proteinen, wijst deze bevinding op een belangrijke rol van de Wnt signaaltransductieweg in verschillende aspecten van foetale ontwikkeling en CDH. In een derde, consanguine familie werd een homozygote mutatie gedetecteerd in PIGN in een

foetus met meerdere congenitale afwijkingen. Deze mutatie werd overgeërfd via elk van beide ouders. PIGN is betrokken in de synthese van 'GPI anchors' wat strookt met de stijgende evidentie dat defecten in de synthese van GPI ankers verschillende fenotypes kunnen veroorzaken.

Aangezien de efficiëntie van de foetale en/of neonatale therapie bij CDH foetussen afhangt van de graad van de hernia en de pulmonaire hypoplasie zou het identificeren van biomerkers die gecorreleerd zijn met de ernst van deze aandoening de therapie kunnen sturen. Daarom besloten we te onderzoeken (i) of de genexpressie in amniosvocht van CDH foetussen een biomarker zou kunnen zijn voor de ernst van de aandoening en het effect van de behandeling.. In deze studie gebruikten we, zoals beschreven in hoofdstuk 6, RNA sequencing om de genexpressie in gekweekte amnioscellen van geïsoleerde CDH foetussen te onderzoeken. Onze analyse identificeerde 2 mogelijke moleculaire subtypes van geïsoleerde CDH, waarvan één gekarakteriseerd is door een verminderde transcriptie van TGFB1 en CTGF en een verhoogde transcriptie van TNF, IL-6 en IL-8. Dit duidt erop dat verminderde transcriptie van TGFB een mogelijke oorzaak is van de geobserveerde gestoorde regulatie van de verschillende doelwitgenen. Daarnaast toont deze groep van patiënten een inflammatoire respons door de verhoogde expressie van TNF, IL6 en IL8 wat postnatale pulmonaire hypertensie kan versterken. Deze resultaten vormen een goede start voor toekomstige studies die in een grotere groep geïsoleerde CDH patiënten zou moeten uitgevoerd worden om de klinische betekenis en het therapeutische potentieel te bepalen.

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